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# THIRD TISSUE HOMOTRANSPLANTATION CONFERENCE*

*Consulting Editor*

BLAIR O ROGERS

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* This series of papers is the result of the *Third Tissue Homotransplantation Conference* held by The New York Academy of Sciences, Section of Biology, February 6 and 7, 1958 See *The Relation of Immunology to Tissue Homotransplantation*, Vol 59, Art 3, based on the conference held by the Academy February 12 and 13, 1954, and *Second Tissue Homotransplantation Conference*, Vol 64, Art 5, based on the conference held by the Academy, February 2 and 3, 1956

## Part III Antibodies and Antigens in Tissue Transplantation



## Part IV Graft-Versus-Host Reaction and Acquired Tolerance in Tissue Homotransplantation

# INTRODUCTORY REMARKS

*By* John Marquis Converse and Blair O. Rogers

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This monograph, based on the third biennial Tissue Homotransplantation Conference, demonstrates, as did the two preceding publications,[1, 2] the rapid advances made in this new biological and clinical specialty: the science of tissue transplantation. We feel that, among the papers presented here, those dealing with specific desensitization of the delayed hypertensive state, evidence of a passive transfer of serum antibody against skin homografts, with further clarification of the role of lymphatics and draining lymph nodes in homotransplantation phenomena, with work now in progress on the identification of the various antigenic components of transplanted cells, and with the increasing attention being paid to the behavior of embryonal, fetal, and neonatal tissue transplants—will undoubtedly have widespread effects in the time which must elapse before the fourth conference is held in 1960.

It is not within the scope of these brief introductory remarks to review the papers that follow. However, it is fair to say that, as a group, they should dispel still further the pessimism regarding any solution to the so-called "homograft problem" that was prevalent several decades ago. It seems probable that these papers will open new avenues of approach to the problem to such an extent that we shall all look forward with eager anticipation to the publication of the next monograph in this series.

The contributions to these pages of our colleagues from Czechoslovakia, Denmark, France, England, and Scotland are particularly welcome. The increasing evidence in our western medical literature of the excellent scientific progress paralleling our own in the biological laboratories of Eastern Europe is especially gratifying.

*References*

1. Ann. N. Y. Acad. Sci. 1955. 59(3): 277–466.
2. Ann. N. Y. Acad. Sci. 1957. 64(5): 735–1073.

# PULSATILE ACTIVITY IN TOTAL TRANSPLANATION OF FETAL MOUSE HEARTS *

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In order to have a means of determining the length of time that individual cells of a homograft may remain viable, we have used the fetal heart as our experimental tissue. The pulsations of the homografted heart, as observed in the transparent chamber, provide a ready means of identification of the transplanted cells and also serve as an unquestionable indicator of their viability. The cessation of the pulsations is a clear-cut end point of the survival time of the graft.

In this paper we present the technique of transplanting fetal hearts and describe the methods employed in their study. We also present observations based on the study of transplanted hearts and data on the survival time of grafted cells. Some preliminary data on the experimental treatment of homografted hearts are also included. While these studies were in progress Poor,[1] at Boston University, Boston, Mass., published a preliminary report on the homotransplantation of fetal hearts in the hamster.

## *Material and Methods*

We employed the Saran window modification[2, 3] of the transparent chamber technique. The beds that were to receive the grafted fetal hearts were prepared in the skin of the mid-dorsum of the mouse exactly as described for grafts of full-thickness skin.[3] Each intact fetal heart was placed directly over a large host vessel lying in the carefully exposed panniculus carnosus. The cellulose acetate ring with a clear, thin film of Saran plastic was then affixed to form the transparent chamber. In a few cases, instead of the plastic window, a "natural window" was prepared by applying an autograft of thin auricular skin to the open bed. About three weeks later the fetal heart was then carefully inserted between the panniculus adiposus of the established autograft of ear skin and the panniculus carnosus of the dorsal skin fold.

The hosts were mice of strains A, C57BR, C57BL, C3Heb, and Swiss albino, and ranged in age from three months to one year.

The transplants were the intact hearts of fetuses obtained from mice on the tenth day to eighteenth day of gestation. In some cases, intact hearts removed from newborn mice one to three days of age were used. The age of the fetuses was estimated from the stages described by Gruneberg.[4] The majority of the fetuses used were from fourteen to seventeen days of age.

To obtain the fetus, a pregnant mouse was sacrificed by cervical dislocation and the uterus was excised aseptically. The fetuses were removed from the

* The work reported in this paper was supported by a grant from the Public Health Service, Bethesda, Md.

uterus, freed of all membranes, and placed on the stage of a dissecting dish Each heart was dissected out rapidly, placed in a balanced salt solution, and then inserted into the transparent chamber A balanced salt solution such as Earle's[5] or Gey's[6] was found to keep the hearts in better condition than did either Ringer's solution or isotonic saline

More than one half of the hearts received no special treatment before or after transplantation However, studies were made on some hearts subjected to freezing and thawing before transplantation and on others perfused with various solutions after transplantation

The following technique was used for freeze-thawing immediately after excision, the heart was placed in a test tube containing 0 2 ml of a solution of 25 per cent glycerol in Morgan, Morton, and Parker's medium No 199[7] that had been prewarmed to 38° C It was then incubated at 38° C for 45 minutes This use of the glycerol solution to protect the cells as much as possible from the damage incurred during freezing was adapted from the work of Smith and Parks [8] According to the findings of Lovelock[9] the glycerol in the cells acts as a "salt buffer"

After incubation the heart was quickly frozen by plunging the test tube into a bath of alcohol and dry ice at −78° C The temperature was carefully maintained and the heart was frozen for 30 minutes The heart was then thawed rapidly by immersing the tube in a water bath at 38° C, and simultaneously rinsing the heart with the mixture of glycerol and medium No 199,[7] which was also at 38° C Immediately after thawing, the heart was inserted into the transparent chamber

The perfusion technique was performed as follows the heart was placed in the transparent chamber immediately after removal from the fetus Twenty-four hours after transplantation, 0 1 ml of an experimental solution was instilled into the chamber once a day for 5 days The experimental solutions which were used were Earle's balanced salt solution,[5] 02 M L-glutamine, and a 10× dilution of Eagle's Vitamin Concentrate Stock B for synthetic media [10]

In all cases, the hearts were observed at least once a day with transmitted light under a stereoscopic microscope

## *Results*

One hundred and sixteen transplanted hearts were studied Eighty-nine were homografts of fetal hearts Of these, 52 were given no special treatment, 18 were perfused after transplantation, and 19 were subjected to rapid freezing and thawing before transplantation Twelve hearts from newborn mice were also homografted In addition, 15 fetal hearts were transplanted as intrastrain or isologous grafts We shall describe the phenomena that were noted in the course of observing the hearts

By the fourth postoperative day, when vascular connections with the hosts were established, all the fetal hearts had resumed beating In general, the rate of pulsation of the homotransplanted fetal hearts remained remarkably constant from almost the fifth to about the sixteenth day Rates of 200 to

300 beats per minute were not uncommon, the normal heart rate of adult mice is 400 to 600 beats per minute. After the sixteenth day the rate gradually diminished and pulsation stopped abruptly at about eighteen days in most cases. Approximately the fourteenth day after homotransplantation the outline of the hearts had become very faint, and it was obvious that the transplants were undergoing absorption. Nevertheless, vigorous pulsations of the transplanted tissue persisted in most cases. In some hearts, however, only one auricle was beating regularly at this time, and the ventricles were contracting intermittently. These findings were interpreted as indicating that, even though a portion of the transplant was evidently necrotic and was undergoing dissolution, there were areas of the grafted tissue that remained viable for a longer period. Histological studies substantiated this interpretation.

Sections of homotransplants made on the fourteenth postoperative day when pulsations were still vigorous showed a pronounced round cell infiltration of the heart. Although the morphologic integrity of the heart was largely obliterated, there remained some semblance of tissue organization and a few areas of the myocardium had a completely normal appearance.

Sections of transplants made as soon as all pulsations ceased showed more diffuse round cell infiltration, and all of the myocardial cells appeared to be necrotic. These findings suggest that, as long as any cells remain viable, they will contract. It is obvious that there is a great advantage in using the heart as the transplant in studies on homograft survival since the viability of even very small areas can be detected.

Twenty-four hearts were fixed for histological studies at various intervals. The 28 remaining homografts of fetal hearts were allowed to continue until all pulsations ceased. The median survival time of the untreated fetal heart homografts was calculated to be 19.3 days.

With a few exceptions, the 18 hearts perfused after homotransplantation and the 19 hearts frozen and thawed before homotransplantation behaved similarly. The median survival time of the perfused hearts was 12.5 days and that of the freeze-thawed hearts was 13.0 days. One homograft treated by freeze-thawing continued to pulsate rhythmically for 35 days. One untreated homograft retained its morphologic integrity and continued to pulsate vigorously for 45 days.

The 12 hearts from newborn mice that were homografted survived for a much shorter period. The median survival time of these hearts was 9.0 days and one graft, although it became visibly vascularized, never resumed beating after transplantation.

The 15 intrastrain grafts of fetal hearts underwent a similar but greatly protracted rejection phenomenon. There were no permanent takes, but 2 of the intrastrain grafts lasted for over 90 days. The calculated survival time for isografts of fetal hearts was 47.0 days.

At no time during our studies of the heart transplants did we observe the regular sequence of events previously described as good criteria for the survival time of skin homografts;[2,3] namely, dilation of blood vessels in the graft, slowing of blood flow, and the formation of thrombi. The median survival time of skin homografts as determined by these criteria was found to be 9.0 days.

*Summary*

In summary, this is the presentation of work in which the hearts of fetal and newborn mice have been transplanted to the dorsal skin fold of unrelated and related recipients This particular tissue has been found to be ideal for the interpretation of the survival time of homotransplanted tissue because the cessation of cardiac pulsations affords a very definite end point Also, the pulsatile activity of a transplanted fetal heart definitely identifies the transplanted tissue from the infiltrating tissue of the recipient Untreated fetal hearts exhibited a median survival time of 19 3 days Fetal hearts that had been perfused showed a median survival time of 12 5 days, those that had been frozen and thawed before transplantation, a median survival time of 13 0 days Homotransplants of hearts from newborn mice 1 to 3 days old had a median survival time of 9 0 days Intrastrain grafts of fetal hearts survived for an average of 47 0 days, and 2 of these grafts exhibited strong spontaneous pulsations for over 90 days The homotransplantation of fetal hearts by means of a tissue-chamber technique is presented here as an ideal method for the assay of the exact effect of experimental pretreatment of homografts on their survival time Other studies are in preparation

*References*

1 Poor, E 1957 Brephoplastic homotransplantation of hamster heart A preliminary report Transplantation Bull 4(4) 143
2 Conway, H, J Sedar & J E Shannon 1957 Re-evaluation of the transparent chamber technique in the study of the circulation in autografts and homografts of skin Transplantation Bull 4(2) 62–64
3 Conway, H, B H Griffith, J Shannon & A Findley 1957 Re-examination of the transparent chamber technique as applied to the study of circulation in autografts and homografts of the skin Plastic Reconstr Surg **20**(2) 103–116
4 Gruneberg, H 1943 The development of some external features in mouse embryos J Heredity **34** 89–92
5 Earle, W R 1943–44 Production of malignancy *in vitro* IV The mouse fibroblast cultures and changes seen in the living cells J Natl Cancer Inst **4**, 165–212
6 Gey, G O & M D Gey 1936 The maintenance of human normal cells and tumor cells in continuous culture I Preliminary report cultivation of mesoblastic tumors and normal tissue and notes on methods of cultivation Am J Cancer **27** 45–76
7 Morgan, J F, H J Morton & R C Parker 1950 Nutrition of animal cells in tissue culture I Initial studies on a synthetic medium Proc Soc. Exptl Biol Med 7 1–8
8 Smith, A. U & A S Parkes 1954 Storage and homografting of endocrine tissues Ciba Foundation Symposium on Preservation and Transplantation of Normal Tissues 76–85 Little, Brown Boston, Mass
9 Lovelock, J E 1954 Biophysical aspects of the freezing of living cells Ciba Foundation Symposium on Preservation and Transplatantion of Normal Tissues 131–140 Little, Brown Boston, Mass
10 Eagle, M 1955 The minimum vitamin requirements of the L and HeLa cells in tissue culture, and the production of specific vitamin deficiencies and their cure J Exptl Med **102** 595–600

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bryo skin, also of full thickness, was usually taken from the backs of the older embryos in pieces as large as possible Skin from the youngest embryos, where much of the material was jellylike in consistency, consisted of minute "blobs" or droplets of material that were spaced (preferably on blood vessels), about 9 to the graft bed Finished grafts were dusted with sulfadiazine powder and covered in turn with vaseline gauze, a transparent bandage, and several gauze pressure pads before they were wrapped with rolled gauze and, finally, with adhesive tape First examination was done 12 to 14 days postoperatively The animal was then rewrapped lightly and examined every week thereafter Bandages were usually kept on for 1 month, after which they were removed completely No difficulty was experienced with destruction of bandaging, provided that the rabbits were caged individually None of the grafts became infected

Frozen skin used for grafting was prepared from adult donors by removing a full-thickness square of skin approximately 7 × 10 cm and folding this piece on itself so that the raw surfaces were together It was then placed in a sterile Petri dish that was sealed with ordinary waterproof adhesive tape and placed in a refrigerator at −4° C until needed Embryos were frozen whole in a similar manner, and the skin was removed after thawing at room temperature The secret of good preservation seemed to lie in not wetting either the adult or embryo skin in any manner before freezing Shaving of the adult skin was done dry with an electric razor, and the embryo was cleaned off, if necessary, with dry sterile gauze Tissues were kept frozen from 1 day to 6 weeks Even after the latter period, they grafted well and compared favorably with fresh adult homografts from the same donor done at the time of freezing or with fresh embryo from the same litter (not on the same recipients) In these experiments only embryos of 18 or more days of gestation were used for freezing, as no extra material was available from the younger groups Skin, adult or embryonic, when kept more than 24 hours at ordinary refrigerator temperature, grew poorly, if at all

The rabbits that were treated with cortisone (Cortone)* received a subcutaneous injection at the time of grafting and a daily injection thereafter except Sundays Animals weighing 3 to 3 5 kg were given an initial dose of 30 mg and a daily dose of 7 to 8 mg Larger animals of 3 5 to 4 0 kg received a primary injection of 35 mg and a subsequent injection of 8 to 10 mg daily Metacortin was used for a series of 20 rabbits, but proved highly toxic to them, usually causing a violent diarrhea, even when only one third to one fourth the cortisone dosage was employed Those animals in which the cortisone treatment was discontinued and ACTH (ACTHAR)† substituted received 2 units of the latter on the first day cortisone was omitted, and 5 subsequent doses of one unit each on alternate days following Two additional doses of 2 mg of cortisone each were injected during the first week of this new treatment on days when ACTH was not given

In general, the health of the rabbits was good A few of the conditioned

* Product of Merck Sharp & Dohme, Philadelphia, Pa
† Product of The Armour Laboratories, Chicago, Ill

# STUDIES OF ADULT AND EMBRYONIC SKIN HOMOGRAFTS ON CONDITIONED OR NORMAL RABBITS, WITH EMPHASIS ON THE POSSIBLE ROLE OF THE GROUND SUBSTANCE*

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This report is based on two years of adventures in homografting at the Surgical Research Unit, Fort Sam Houston, Texas During this period, over 1000 adult rabbit recipients and donors were used, including 76 pregnant mothers and their embryos, in order to find some method of prolonging homograft survival Three hundred rabbits alone were employed in a series of desensitization studies that were at best suggestive and will not be reported here From the welter of statistics and data accumulated on the remainder there emerged 4 salient positive facts that seem worthy of note These are reported in this paper

## MATERIAL AND METHODS

The rabbits used were young adult females of colored (gray chinchilla, Red Satins, Black Dutch) or New Zealand White stock obtained from a commercial breeder These animals averaged from 3 to 4 kg in weight, a few being heavier or lighter For convenience in caging, only female animals were employed

Pregnant mothers were either obtained as such from the breeder or bred in the laboratory by allowing them to remain with the male for one 6-hour period only The exact age of the embryo could be obtained by the latter procedure When the mothers were aborted records were kept of the embryo measurements so that a fairly good estimate could be made of the age of those embryos obtained from does where the gestation period was unknown by comparing them with embryos of determined age Allowance in size was made for the number of animals per litter In general, 4 age groups of embryos were employed 14 days or under, 16 to 19 days, 23 to 26 days, and 28 to 30 days Full term for rabbits is 32 days

All rabbits, including the pregnant mothers, were anesthetized with paraldehyde injected intramuscularly (approximately 3 ml in each hind leg for the larger animals and 2 5 ml for the smaller) Graft beds were prepared on the chest wall in the manner described by Medawar[1] For adult homografts an area approximately 10 × 7 cm was marked off with a scalpel, and the demarcated epidermis and dermis were stripped off with this same sharp blade The muscularis, with its overlying fascia and vessels, was thus exposed and used as the graft bed The area prepared for the reception of skin from the very young embryos was smaller, due to lack of material, and in general measured 6 × 7 cm

Adult homografts were placed on the recipient as full-thickness strips of skin measuring about 5 × 1 cm Five to six strips were used per graft Em-

* The work reported in this paper was performed at the Surgical Research Unit, Fort Sam Houston, Texas

case it also preserved and grafted successfully when frozen, and was universally accepted by various conditioned recipients, on some of which grafts of thick skin, simultaneously done, would not take at all

The latter finding indicates that some mechanical factor other than rejection due to immune processes was responsible for the graft failure Certainly, it is possible that the graft may not "take" for reasons completely unrelated to an active immunity Intensive pretreatment of a host with cortisone, for example, may so damage the fibroblast and vascular response of the graft bed that no adherence or vascularization of the donor skin strips is possible, they thus "slough," yet at no time has the host actually "rejected" this graft Such possibilities must be borne in mind in the evaluation of a graft series *

It is noteworthy that the adult homografts on the conditioned hosts seldom grew hair At best only a few isolated hairs, different in color from those of the host, were seen At the same time the area of host skin that had been shaved when the graft bed was prepared often remained bare This inhibitory effect of the adrenal hormones on hair growth has been well documented [3, 4] In contrast, the hair on skin derived from an embryo of any age appeared within 2 weeks after grafting, was most luxuriant, and apparently remained unaffected by the cortisone treatment

### *Survival of Very Young Embryo Skin on Untreated Hosts*

Although none of the adult or older embryo homografts survived on untreated rabbits, 45 per cent of the skin from embryos of 12 to 13 days' gestation became permanent grafts on such normal hosts † Also of interest was the finding that these grafts sloughed in half of the conditioned rabbits

TABLE 1 summarizes the experiments done with this young tissue Ten rabbits were grafted with the embryos from 4 mothers known to be 13 days pregnant Seven of the recipients were treated with cortisone, 3 were not All of the latter grafts became permanent in the sense that they survived 3 months or more until the host died or was sacrificed Of the 7 grafts on conditioned animals, 2 became greatly swollen and edematous—so much so that they resembled hair-covered pink water bags These 2 grafts were very friable and soon rubbed off and were replaced with host tissues In another group of experiments in which 12-day embryos from 14 pregnant mothers were

* In a group of 18 rabbits pretreated in 3 sets of 6 animals each for 1, 4, and 7 days, respectively, prior to grafting, only those pretreated for 1 day were as successfully grafted as an additional 6 rabbits not given cortisone until immediately after the operation (the usual procedure)

In another experiment 3 sets of "round-robin" grafts (4 rabbits to a set) were devised, wherein 2 rabbits were pretreated with cortisone prior to grafting and two were not All were conditioned after grafting The experiment was so arranged that the skin from rabbits 1 → 2 → 3 → 4 → 1 It was learned that there was no advantage in placing pretreated skin on either pretreated or previously untreated hosts, nor did untreated skin do as well on the pretreated hosts as the standard untreated skin on previously untreated animals

† Embryos of this age are so tiny, varying in length from 1 cm to 0 7 cm or less, that at best 2 rabbits can be grafted with the embryo skin from one pregnant doe The skin is very fragile and transparent and must be put on the graft bed as minute dabs approximately 1 to 2 mm in width However, the proliferative capacity of these tissues is extreme for, in 3 weeks' time, such small pieces can become 2 cm square

hosts reacted strongly to the cortisone and developed a diarrhea or pneumonia, the latter, which was usually due to a combination of *Proteus vulgaris* and pneumococcus, could often be controlled with penicillin and Chloromycetin Edema was not common in spite of the extensive cortisone treatment, possibly because the Purina rabbit chow* used for feeding had a low salt content (carrots were given also twice weekly) Accidents such as broken legs, which were usually caused by a reaction to barking dogs in the adjacent room, and excessive heat accounted for the death or necessary destruction of a number of animals

All rabbits were weighed twice weekly and a differential and total blood count was made once weekly Every attempt was directed toward the maintenance of a steady weight If any animal showed a 0 2-kg loss in a 7-day period the daily cortisone dosage was cut until no further loss occurred

It should be noted that the color of the donor animals, whether adult or embryo, was always different than that of the recipient host, that is, grafts from white rabbits were placed on black, red, or gray chinchilla hosts and vice versa Grafts that grew hair were thus labeled by visible hair color or, when sectioned, could be identified by the hair follicles (colored or white)

## Results

### *Prolonged Survival of Adult and Embryo Homografts in Cortisone-Treated Hosts*

The first finding of note in these studies was that full-thickness homografts of adult or embryo skin (16 days' gestation or more) did so well in properly cortisone-treated hosts that they simulated autografts, whereas neither survived in the 75 untreated hosts that were grafted Almost the only difficulty encountered in attaining permanent maintenance in conditioned animals was that of finding the correct cortisone dosage for any given rabbit A delicate balance existed between the amount of treatment needed to produce a susceptible state in the host and that which would result in a sick animal In some rabbits the two situations overlapped Such animals were so susceptible to the treatment that they died or were sacrificed before much time had elapsed However, if the individual dose could be adjusted (on the basis of weight loss and general health) for any one rabbit, the grafts were well maintained Indeed, 20 animals were treated for as long as 6 months, remained in relatively good health, and had intact and excellent grafts at the end of this period This is in contrast to the generally accepted idea that skin homografts in cortisone-treated rabbits usually last about 3 to 4 weeks at best[2] Twelve of the 20 were adult skin grafts, the remainder were embryo skin aged approximately 18, 24, or 30 days The proportion is not relative to the total number of adult versus embryo grafts done, but to the number of animals saved, since many of the hosts with grafted embryo skin were used for other purposes before the 6-month period elapsed In general, 16- to 31-day-old embryo skin, whether fresh or frozen, always grafted well, while homografts of adult skin were comparable to the embryo grafts only if the skin was thin In the latter

* Product of Purina Mills, Buffalo, N Y

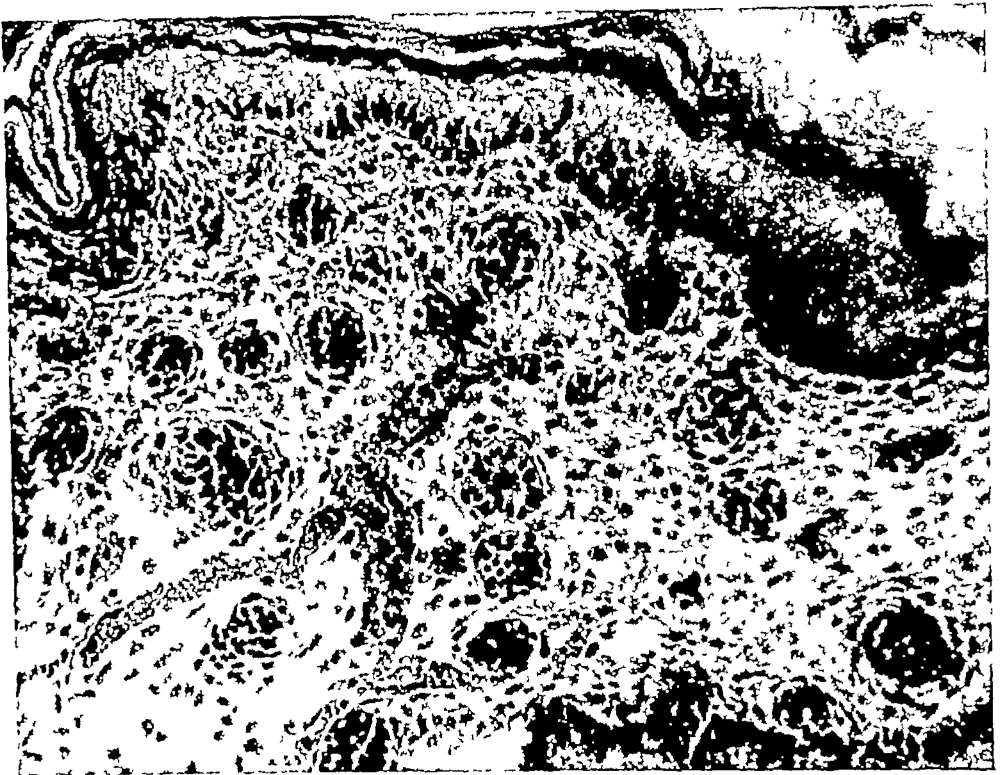


FIGURE 2 Fresh skin from a 21-day-old embryo after 8 days on an adult cortisone-treated host There has been extensive proliferation in the dermis as well as in the epidermis, and there are many plump fibroblasts Numerous blood vessels are present ×170

were all similar in these respects there had been little maturation of the formed elements of the connective tissue ground substance, such as collagen, the fibroblasts were few in number, the blood vessels, though numerous, were thin walled, and the epidermis itself tended to be somewhat scanty Hair, however, was abundant (FIGURE 1) In sharp contrast, in skin grafts from older embryos, plump fibroblasts soon became evident and active (FIGURE 2), and there was rapid maturation of both epidermal and dermal elements (FIGURE 3)

### *"Slow-Motion" Rejection of Grafts on Conditioned Hosts After Cessation of Cortisone Treatment*

Previous studies that have been done on skin homograft rejection have dealt with the rapid slough seen in the untreated host after a brief period of acceptance The usual procedure has been either to take biopsies at stated intervals in the week to 10 days that encompass the initial vascularization and final rejection, or to study a graft *in toto* by means of "chamber" techniques From these observations a general impression has evolved of an overwhelming lymphocytic infiltration of the graft and/or a stasis in the graft blood vessels

It was therefore of interest to learn that homografts on rabbits maintained for long periods on cortisone would take 3 weeks to 1 month to slough after

TABLE 1

| Age of embryo | Number of rabbits grafted | Conditioned with cortisone | Successful with cortisone | Untreated | Successful untreated |
|---|---|---|---|---|---|
| 13 days | 10 | 7 | 5 | 3 | 3 |
| 12 days | 30 | 13 | 5 | 17 | 6 |
| Totals | 40 | 20 | 10 | 20 | 9 |

grafted on 30 recipients, 13 of the latter were conditioned and 17 were not Six of the 17 grafts on normal animals became permanent Eight of the grafts on cortisone-treated animals again became very edematous and were lost The grafts that took on untreated hosts were quite flat, those that sloughed did so without swelling and in the usual well-documented pattern It should be noted that the slough of the edematous grafts on conditioned rabbits was not considered an active rejection by the host, that is, a slough in the usual sense Rather, it was thought to be a manifestation of the immaturity or defectiveness of the graft ground substance that was unable to handle the biophysical problems imposed by cortisone administration

Histological sections of the material grafted showed very immature skin, with 2 layers of epithelial cells, no hair primordia, and an undifferentiated dermis Microscopic examination of the successful grafts from these tissues

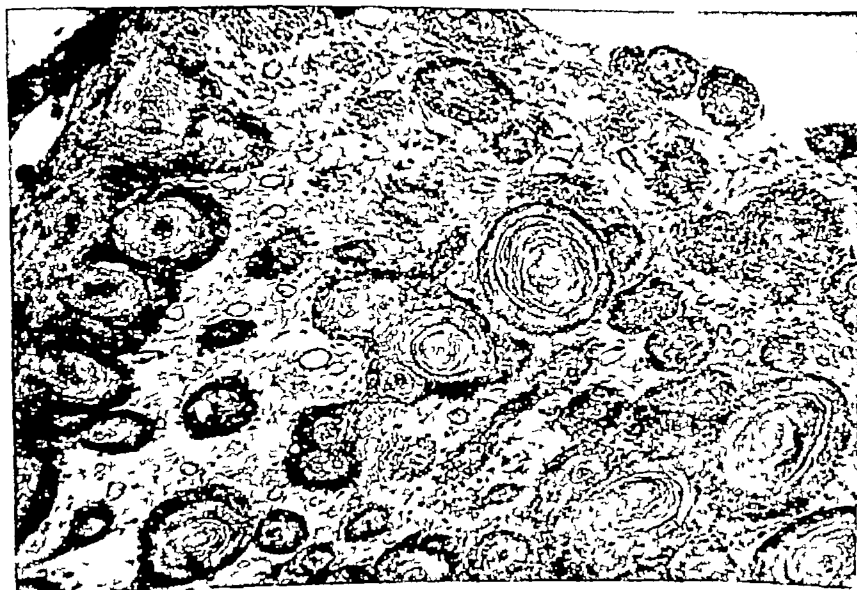


FIGURE 1 Skin from a 12-day-old embryo that had grown for 2 months on an untreated adult host It is notable for its lack of formed connective components such as collagen and for the paucity of fibroblasts The epidermis is quite thin, but it appears to be healthy There are many capillarylike blood vessels ×105

removed as a whole, cut into 6 pieces across the whole width, and sections made of these entire strips No biopsies were ever taken, as it was felt that an inflammatory reaction to such a procedure that would obscure the findings might occur The strip was studied as a unit because changes often occurred in one region while others were still unaltered Biopsies of any one of these areas would have given a very false impression of the whole

When all these sections were examined it could be seen that a progression of events had occurred, a type of "slow-motion" picture of graft rejection Whether the process observed is similar to the rapid slough seen in untreated hosts and is merely kaleidoscoped is, of course, unknown There were no essential differences in grafts derived from adult or embryo sources

*Sequence of Events, as Seen Microscopically, in the "Slow" Rejection*

The microscopic changes seen in the grafts described above were similar to those observed in grafts of several animals given insufficient cortisone after an initial adequate dose The sequence of events will thus be described as one The first alteration noted in an otherwise healthy-looking graft was a hyalinization of the ground substance, without the influx of any host cells whatsoever or any visible change in the blood vessels (FIGURES 4 and 5) In some of the grafts examined, only the areas adjacent to the host tissues had



FIGURE 4 Fresh skin from a 25-day-old embryo after 18 days on an inadequtely conditioned adult host Although the initial growth was evidently excellent, this graft is showing early hyalinlike changes in the dermal tissue The blood vessels still appear to be normal, and there are no accumulations of host cells ×105

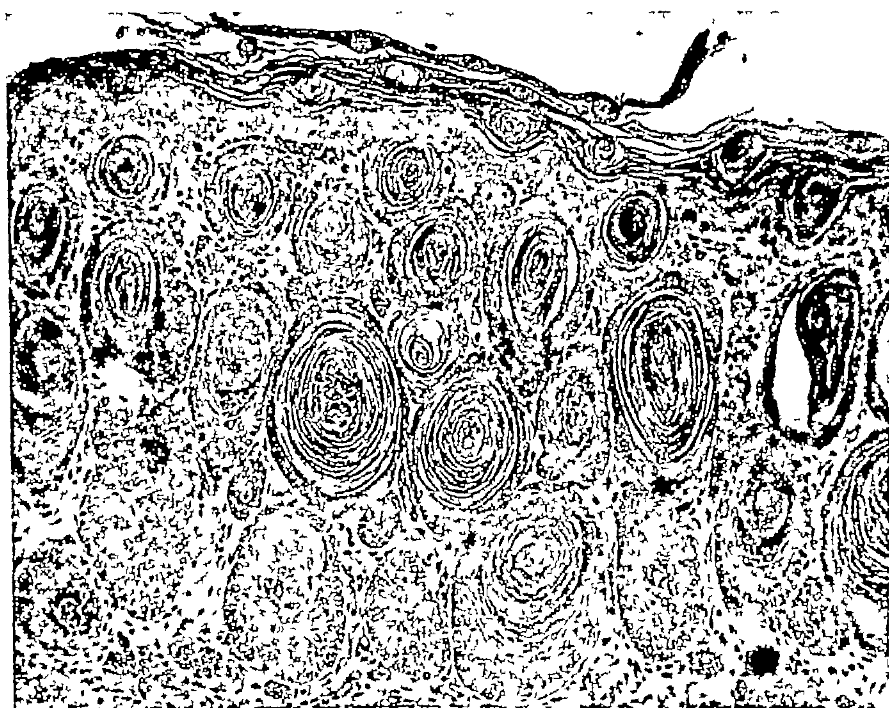


FIGURE 3 Skin from the same group of 21 day-old embryos as that in FIGURE 2 However, it was first frozen at −4° C for 4 days before being grafted onto an adult cortisone treated host This specimen has been allowed to proliferate for 14 days before removal, it grew just as well as the fresh embryo material. ×105

cortisone was discontinued During the interval without treatment of the host the graft appeared to be in good condition Indeed, there was often a question (in the case of hairless adult grafts) as to whether the host tissues had replaced the donor skin Eventually, however, the graft would discolor, usually beginning to do so at one of the edges adjoining the host tissue Within a few days thereafter a complete slough occurred It was felt that it might be possible to learn from this delayed rejection whether there were changes taking place in the graft that were usually obscured in the "quick" slough so often described Therefore, a total of 30 animals with good grafts of 2 months' duration or longer (including 15 of the 20 kept for 6 months) were taken off cortisone and given a small amount of ACTH* to sustain them for the first week They were either kept until their grafts sloughed completely (6 animals) or sacrificed at intervals (4 rabbits every fourth day) Each graft was

* ACTH is not effective in prolonging the life of skin grafts in rabbits This is probably due to the fact that the major adrenal hormone excreted by these animals is compound B or corticosterone,[5] which is also without effect on such grafts In any transplantation work with conditioned animals it is important to know the adrenal steroid pattern of the host chosen Results obtained with ACTH or certain adrenal steroids in one species cannot always be transposed to another

removed as a whole, cut into 6 pieces across the whole width, and sections made of these entire strips No biopsies were ever taken, as it was felt that an inflammatory reaction to such a procedure that would obscure the findings might occur The strip was studied as a unit because changes often occurred in one region while others were still unaltered Biopsies of any one of these areas would have given a very false impression of the whole

When all these sections were examined it could be seen that a progression of events had occurred, a type of "slow-motion" picture of graft rejection Whether the process observed is similar to the rapid slough seen in untreated hosts and is merely kaleidoscoped is, of course, unknown There were no essential differences in grafts derived from adult or embryo sources

*Sequence of Events, as Seen Microscopically, in the "Slow" Rejection*

The microscopic changes seen in the grafts described above were similar to those observed in grafts of several animals given insufficient cortisone after an initial adequate dose The sequence of events will thus be described as one The first alteration noted in an otherwise healthy-looking graft was a hyalinization of the ground substance, without the influx of any host cells whatsoever or any visible change in the blood vessels (FIGURES 4 and 5) In some of the grafts examined, only the areas adjacent to the host tissues had

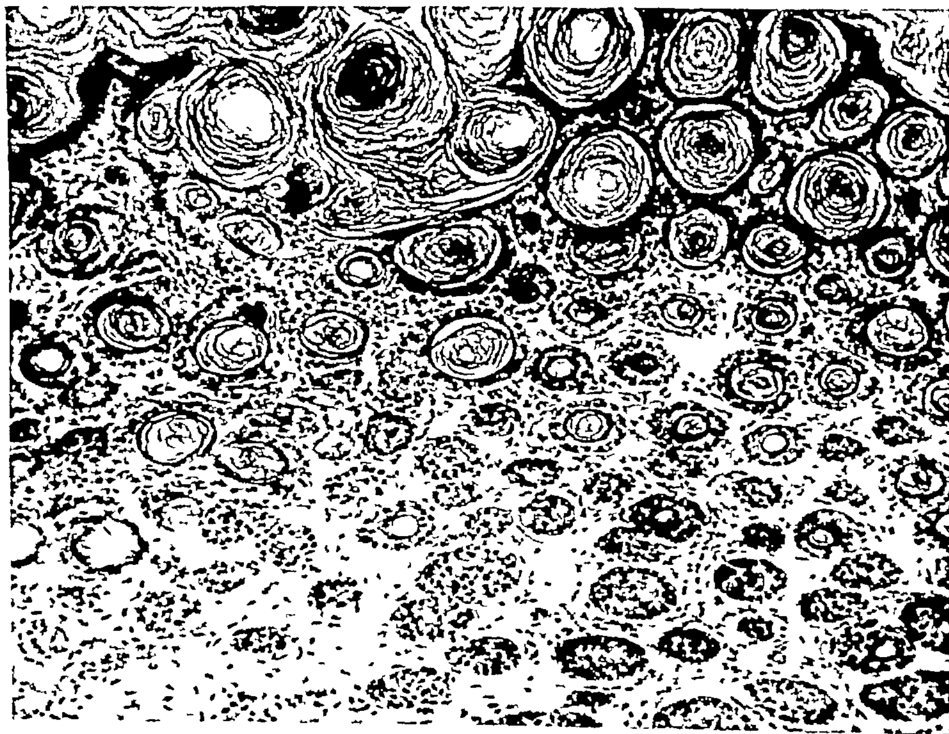


FIGURE 4 Fresh skin from a 25-day-old embryo after 18 days on an inadequately conditioned adult host Although the initial growth was evidently excellent, this graft is showing early hyalinlike changes in the dermal tissue The blood vessels still appear to be normal, and there are no accumulations of host cells ×105



FIGURE 7 Same slide as in FIGURE 6, showing migration of host polymorphonuclear cells through the epidermis, which remains in good condition A crust (arrow) is formed on the graft exterior by the emigrated dead host leukocytes ×475

is a multipotential substance that not only affects cells associated with phagocytosis,[10 12] antibody production,[13] and allergic responses[14] but, among other things, has a great effect on the connective tissue ground substance itself [15] It may act on the latter by its depression of such cells as fibroblasts, which are correlated with the production of collagen and possibly other formed elements, and/or mast cells, which have been associated with mucopolysaccharides [16] Also, it may affect the ground substance more directly by inhibiting the synthesis of chondroitin sulfate[17] and by altering the amount and chemistry of such substances as hyaluronidase, possibly through sulfhydryl deprivation [18 19] It is also known to alter the migration of leukocytes from the blood vessels and the formation of fibrin and edema [20 23] Numerous additional effects of the adrenocortical hormones have been reviewed elsewhere [16 24 25] Some of these results of cortisone administration obviously may act on a graft in purely mechanical fashion For example, such a graft may fail to take if the supportive tissue or vascular response has been rendered nearly inert by intensive preconditioning Indeed, any dozen of the manifold effects of cortisone may jeopardize the success of grafting without in themselves being referable to resistance factors However, in the grafts described herein there was

FIGURE 8 Skin from the same group of embryos as in FIGURES 6 and 7 on a host that had been given inadequate cortisone treatment after the initial dose This graft is 31 days old Lymphocytes have appeared and, wherever they have become associated with the epidermis, lysis of the graft cells has occurred Areas of epidermis without lymphocytes are still in good condition ×475

FIGURE 9 Higher magnification of slide shown in FIGURE 8 ×760



FIGURE 10 Thin homograft 75 days after grafting on a conditioned host Cortisone had been discontinued 15 days previously and ACTH given on alternate days thereafter The graft still appeared to be in good condition on the rabbit This slide, however, shows hyalin changes in the connective tissue and, in color, outlines beautifully the polymorphonuclear leukocytes in the epidermis and throughout the left side of the graft The cells that have just arrived in the tissues to the right are lymphocytes (arrow) The epidermis appears to be in good condition, there are as yet no lymphocytes associated with it ×105

good attachment to the host, and it would appear that the survival resulted from a true abrogation of resistance phenomena However, it is not at all certain at what level such resistance was inhibited

Much attention currently paid to the factors underlying host resistance to foreign tissues has centered on the role that the lymphocyte may play The beautiful experiments of Algire,[26] Mitchison,[27] and Billingham *et al*,[28] among others, have served to spotlight this cell, yet the experiments reported herein indicate that the ground substance may also deserve some consideration Certainly, the first changes seen when the host became immunologically active were in the connective tissues of the graft Had the cortisone prevented previous changes therein, either because it altered or inhibited elements of this tissue per se, or the immunological responses to them or, possibly, both? Was the graft connective tissue the site of an "antigen-antibody" reaction, with entities of the ground substance providing the antigen? This latter idea becomes attractive in view of the fact that some of the very young embryo tissue was able to grow permanently in a number of untreated hosts in which the immunological system was intact and unhampered Was no antigen provided

because the ground substance of the embryo was as yet unformed or not integrated? That administration of cortisone actually caused a tremendous edema in several of these young grafts may also indicate how ill equipped their connective tissues were to handle stress of any kind On the other hand, the possible "nonantigenicity" of the young embryo (if such it is) and the reactions seen in older grafts may bear no relation to one another

These thoughts are at present no more than speculations However, the progression of events described in the slowly rejected grafts, and the lack of formed connective tissue persisting in the embryo grafts that became permanent in untreated animals, would seem to warrant consideration of the ground substance in graft rejection, whether it prove of major significance in itself or a minor manifestation of a large-scale process

## Summary

It was learned that both fresh and frozen homografts of adult or embryo skin could be maintained indefinitely on rabbits properly conditioned with cortisone When treatment of these hosts was discontinued the grafts sloughed in 3 weeks to 1 month in a type of slow-motion rejection, in contrast to the violent and rapid slough seen in unconditioned animals Histological study of a series of grafts from such previously cortisone-treated hosts revealed a characteristic progression of events

First, there was a hyalinization of the ground substance without the influx of any host cells Second, polymorphonuclear leukocytes invaded the graft from the local blood vessels and migrated out through the epidermis without any visible effect on it Some of the leukocytes, however, adhered to the hair shafts and base of the epidermis, where they became greatly swollen After this migration the lymphocytes arrived and lysed the epithelium, and a holocaust of degeneration followed These same events also occurred in grafts in inadequately conditioned rabbits

Correlated with these findings was the observation that the only skin grafts retained by unconditioned hosts were derived from very young embryos late in the first trimester of gestation Such grafts always remained deficient in connective tissue elements even though they appeared to be in good condition on their adult hosts On cortisone-treated animals they did poorly and often sloughed after first becoming greatly edematous, again probably due to a defect in the dermis

It is concluded that the ground substance and its possible role in graft rejection or acceptance merit the attention of students of transplantation phenomena

## Acknowledgment

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## References

1 Medawar, P B 1944 The behaviour and fate of skin autografts and skin homografts in rabbits J Anat 77. 176–199

2 Billingham, R E, P L Krohn & P B Medawar 1951 Effect of cortisone on survival of skin homografts in rabbits Brit Med J 1 1158–1163

3 BUTCHER, E O 1937 Hair growth in adrenalectomized and adrenalectomized thyroxin-treated rats Am J Physiol 120 427–434
4 CHASE, H B 1954 Growth of the hair Physiol Revs 34 113–126
5 BUSH, I E 1953 Species differences in adrenocortical secretion J Endocrinol 9 95–100
6 TOOLAN, H W 1957 Growth of embryonic gut and stomach on the exterior chest wall of adult cortisonized hosts Cancer Research 17 707–710
7 TOOLAN, H W 1953 Growth of human tumors in cortisone-treated laboratory animals the possibility of obtaining permanently transplantable human tumors Cancer Research 13 389–394
8 TOOLAN, H W 1954 Growth of human embryonic tissues in cortisone treated laboratory animals Proc Soc Exptl Biol Med 86 607–612
9 TOOLAN, H W 1955 Subcutaneous growth of normal and malignant human tissues in heterologous hosts Trans N Y Acad Sci Ser II. 17(8) 589–593
10 NICOL, T & R SNELL. 1954 Cortisone in relation to infection and tumor growth Nature 174 554
11 CREPEA, S, G MAGNIN & C SEASTONE 1951 Effect of ACTH and cortisone on phagocytosis Proc Soc Exptl Biol Med 77 704–706
12 HELLER, J 1955 Cortisone and phagocytosis Endocrinology 56 80–85
13 BJORNEBOE, M, E FISCHEL, & H STOERK 1951 The effect of cortisone and adrenocorticotrophic hormones on the concentration of circulating antibody J Exptl Med 93 37–48
14 PADAWAR, J & A GORDON 1952 A mechanism for the eosinopenia induced by cortisone and by epinephrine Endocrinology 51 52–57
15 RAGAN, C, E HOWES, C PLOTZ, K MEYER, & J BLUNT 1949 Effect of cortisone on production of granulation tissue in the rabbit Proc Soc Exptl Biol Med 72 718–721
16 THE EFFECT OF ACTH AND CORTISONE UPON INFECTION AND RESISTANCE 1953 Gregory Schwartzman, Ed Columbia Univ Press, New York, N Y
17 LAYTON, L L 1951 Effect of cortisone upon chondroitin sulfate synthesis by animal tissues Proc Soc Exptl Biol Med 76 596–598
18 SEIFTER, J, D BAEDER & A DERVINIS 1949 Alteration in permeability of some membranes by hyaluronidase and inhibition of this effect by steroids Proc Soc Exptl Biol Med 72 136–141
19 ANDERSON, G, L WIESEL, R HILLMAN & W STUMPE 1951 Sulfhydryl inhibition as a mechanism in the effects of ACTH and cortisone Proc. Soc Exptl Biol Med 76 825–827
20 MICHAEL, M & C WHORTON 1951 Delay of the early inflammatory response by cortisone Proc. Soc Exptl Biol Med 76 754–756
21 DOUGHERTY, T & G SCHNEEBELI 1950 Role of cortisone in regulation of inflammation Proc. Soc Exptl. Biol Med 75 854–859
22 EBERT, R. 1952 *In vivo* observations on the effect of cortisone Am Rev Tuberc 65 64–74
23 MOON, V & G TERSHAKOVEC. 1952 Influence of cortisone on acute inflammation Proc Soc Exptl Biol Med 79 63–65
24 KASS, E & M FINLAND 1953 Adrenocortical hormones in infection and immunity Ann Rev Microbiol 7 361–388
25 THE HUMAN ADRENAL CORTEX. 1955 Ciba Foundation 8 Little, Brown Boston, Mass
26 ALGIRE, G, J WEAVER & R PREHN 1957 Studies on tissue homotransplantation in mice, using diffusion-chamber methods Ann N Y Acad Sci 64(5) 1009–1013
27 MITCHISON, N 1955 Studies on the response to foreign tumor implants in the mouse J Exptl Med 102 157–198
28 BILLINGHAM, R., R. BRENT & P MEDAWAR 1953 Actively acquired tolerance of foreign cells Nature 172 603–606

# CLINICAL APPLICATION OF EMBRYONIC SKIN GRAFTS

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Impressed by the work of Helene Toolan on embryo grafts in animals, some of which she reports elsewhere in this monograph, I thought it worth while to attempt to duplicate her work in humans I did not appreciate the problems of duplicating this work when I undertook this project I planned to collect a large number of cases in a short period of time, but obtaining human fetuses proved difficult The many problems of procuring such material need not be discussed here I may say, however, that anyone undertaking such work finds himself deeply involved in the laws and legal opinions of the community in which he works

The second problem is that of obtaining suitable patients for grafting when fetal skin becomes available

The fetuses used in this study have all been less than one half the period of gestation, that is, less than four and one half months of age This age limit was adopted at the suggestion of Toolan and follows her results with animals

The embryos are obtained as shortly after removal as possible, and the skin is removed under sterile conditions It is taken off as full-thickness skin and can be easily peeled away from the deeper structures It appears almost transparent, the blood vessels are easily visible through it The amount of usable skin obtained from an embryo less than 3 months of age is rather small, but a 4-month fetus will supply 30 to 40 $cm^2$ if dissection is performed carefully The sections of skin are placed in a sterile sponge moistened with Ringer's solution and then placed in a sterile Petri dish If the skin cannot be used at once it is stored at 4° C

To date the grafts have been used in 2 groups of patients One group consists of patients with large open wounds, for example, those with burns or granulating areas from skin sloughs or those undergoing major reconstructive surgery The other group consists of volunteers

In the second group of patients a full-thickness defect is created on the forearm of the patient This defect is the size of a United States half dollar The graft is cut to size and sutured into position with long black silk sutures A dressing is tied into position with these sutures This allows for careful immobilization of the graft A successful fetal skin graft appears almost transparent at first It slowly increases in texture and color

To date we have used 5 embryos in our study These embryos have been between 2 and 4 months of age The grafts have been placed on 9 recipients The following is a brief description of each graft

*Patient No 1* Diagnosis multiple myeloma Surgical defect was created on the left forearm on February 27, 1957 Graft still intact eleven and one half months later

*Patient No 2* Diagnosis malignant melanoma Postage-stamp grafts were applied to ulcer on leg February 2, 1957 Grafts took and slowly spread out to heal ulcer Area healed eleven and one half months later

*Patient No 3* Diagnosis radiation necrosis of skin Graft applied to donor area of flap while patient was undergoing reconstructive surgery The wound became infected and the graft was lost

*Patient No 4* Diagnosis second- and third-degree burns Postage-stamp grafts applied to granulating tissue All grafts lost because of infection

*Patient No 5* Diagnosis carcinoma of bladder Postage-stamp grafts applied to granulating abdominal wall wound All grafts lost because of infection

*Application No 6* (to a dog undergoing experimental surgery) Graft applied to surgically created defect in the side of the animal, 100 per cent take at end of 7 days Unfortunately, the dog licked the graft off 3 days later

*Patient No 7* Diagnosis reticulum cell sarcoma Graft applied to surgically created defect left forearm on January 15, 1958 Graft still present and viable 21 days later

*Patient No 8* Diagnosis reticulum cell sarcoma Graft applied to surgically created defect left forearm Graft lost because of infection

*Patient No 9* Diagnosis 50 per cent third-degree burns Skin from 2 embryos applied as postage-stamp grafts on January 30, 1958 Graft still present on February 6, 1958

Of 8 patients grafted to date, 4 have maintained the embryo grafts Of these four, 2 patients have maintained them nearly 1 year The grafts that were lost had never taken and were lost because of infection

To those who work chiefly in laboratories and can control their investigations, I hasten to admit that this work is deficient in many aspects However, it must be remembered that I have been dealing with human patients in whom my work is but a small part of the treatment

The following defects in this work are obvious

(1) It is impossible to be sure of the exact age of the fetuses However, all were below four and one half months of age

(2) There is no absolute way to show that a fetal homograft has survived After 6 to 8 months the surgical wound has contracted and it is difficult to tell whether the graft is present One graft was biopsied 6 weeks after application and was reported as fetal skin In the future it is hoped that female fetal skin can be grafted on male patients, and that identification of sex chromosomes can be used as an indication of survival

(3) Most of our patients have been afflicted with neoplastic disease At present I am carrying out work that shows that these same patients support adult skin homografts for a longer period of time than do normal persons The blood properdin level in these patients has been low It would be interesting to carry out fetal grafts on normal persons

In spite of these criticisms, I believe that the work should be continued and improved Since no one institution can ever have a large number of fetuses with which to work, it would be worth while if many institutions would carry out such experiments

I feel that in many cases the work of the laboratory should be brought to the patient much sooner than is frequently done While there are many prob-

lems and many details to iron out, I believe that the application of Toolan's work in animals to human patients will aid in the understanding of the homograft problem

It is difficult to visualize a supply of fetal skin that would be large enough to be of great clinical importance, yet in the case of a severely burned child the application of fetal skin may be life saving

In conclusion, it appears that human embryo skin less than four and one half months of age will survive permanently when transplanted to an adult It may be postulated that embryonal skin is unable to produce in the recipient a response that causes the graft to be rejected

# FETAL TISSUE HOMOGRAFTS*

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The transplantation of fetal tissue into young or adult animals has been performed for at least three quarters of a century A few embryologists[1-5] published papers many years ago and, more recently, fetal homografts on various laboratory animals have been described by numerous investigators [6-15]

During the past few years this research has been extended to include the transplantation of fetal homografts in humans Gaillard,[16] Helsingen and Helsingen[17] and Toolan[18] have described interesting findings, but their conclusions vary Willis[5] very concisely summarized the results of animal fetal homografts up to 1935, and his deductions still appear to be valid He stated "An embryo or parts of embryos implanted into adults of like species may suffer any of three fates

1 The tissues may be rapidly and completely absorbed

2 They may survive, grow and differentiate for some days or weeks, and then suffer absorption

3 They may grow to considerable masses of mixed differentiated tissues which may persist for long periods, even for the lifetime of the host animal "

Why should this disparity of results be present? The potentiality for survival with regard to the fetal graft may vary with the species, age, viability of the tissue, and the type of tissue used With regard to the recipient, it may vary with age, site of implant, health of the host, and genetic relationship

This investigation was undertaken to determine the average length of survival of human fetal homografts, with special reference to skin transplantation It has been claimed that this type of graft will persist for many months Confirmation of this belief would be of great clinical importance since the homografting of skin and subsequent care of patients with very extensive third-degree burns would be greatly simplified The study was divided into two parts, the first was the use of skin obtained from human fetuses over sixteen weeks' gestation, and the second was the homoimplantation of skin and other tissues from fetuses under sixteen weeks' gestation

The reason for this arbitrary division was that skin from fetuses over sixteen weeks of age was sufficiently durable to withstand the rigors of surface grafting, while that from fetuses under sixteen weeks was so gelatinous and friable that the end point of homograft rejection could have been markedly influenced by other adventitious variables such as infection and surface trauma Therefore, the younger tissues were implanted subcutaneously as homoimplants and heteroimplants

The fetuses used were obtained following premature delivery and spontaneous and therapeutic abortions The age was calculated from the weight,

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foot-length, and crown-rump length The skin was removed as soon as possible after death and stored at 4° C until used These grafts were applied to patients undergoing reconstructive procedures when fresh raw surfaces were temporarily available A pressure dressing was applied and changed at regular intervals All grafts took initially, and the duration of graft survival was determined clinically by loss of epithelium This stage of the rejection phenomenon was used as the end point since it indicated termination of the functional utility of the graft

A total of 17 full-thickness fetal skin homografts was performed in this series (TABLE 1) Grafts from 8 male and 4 female fetuses were applied to 5 male and 2 female recipients The latter varied in age from 1 to 64 years Major blood group (A, B, O, Rh, M, and N) determinations were performed on 10 of the 12 fetuses and on all recipients

The shortest interval between fetal death and removal of the skin was 4 hours, with skin grafting carried out 2 hours later, the remaining 16 grafts were stored from 3 to 20 days It is apparent from our results that if the skin was removed within 24 hours and stored for less than 3 weeks, these variants did not appear to affect the length of survival of the grafts

None of the blood groups of the fetuses was identical with those of the recipients of the skin grafts Other investigators have reported instances of homografting of skin between patients with complete matching of all known blood groups without permanent survival [19, 20]

Survival time of the grafts varied from 4 to 46 days (average, 12 6) Statistical analysis of this small group of cases revealed no correlation between survival time of the skin graft and age of the fetus between 17 and 33 weeks Previous investigators have claimed that younger fetal skin grafts survived longer than older ones because the former were less antigenic However, the graft that survived for the longest period was obtained from a 33-week-old fetus Furthermore, there was no difference in survival if the donor and recipient were of like or opposite sex Finally, similarity or dissimilarity of the blood groups determined did not affect length of survival of the skin grafts

The second phase of this experiment, the implantation of tissues from fetuses under 16 weeks gestation, was undertaken recently to determine if such grafts survived for prolonged periods or even permanently If these tissues did not evoke a homograft reaction, then the differences in protein structure between it and adult skin might be shown by means of comparative studies

Six human fetuses have been obtained for study These varied in age from 7 5 to 13 weeks All tissues used were implanted within 24 hours after death, with an average of 7 hours The tissues were cut into sheets with a maximum thickness of 1 mm and from 2 to 5 mm in diameter They were implanted subcutaneously to obtain protection from trauma, to provide a maximal blood supply and to minimize the risk of bacterial contamination

Fetus No 1, aged 7 5 weeks, was divided into small pieces of mixed tissue and heteroimplanted subcutaneously and intrahepatically into an adult rabbit Several biopsies from different sites were performed The last viable biopsy from the subcutaneous site was obtained 29 days after implantation and

TABLE 1

| Donor Fetus | | | | | | Recipient | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No | Sex | Gestation, age (weeks) | Wt. (grams) | Blood type | Time of storage (days) | No of graft | No of recipient | Sex | Age (years) | Blood type | Sur vival (days) | Observations |
| 1 | Male | 31 | 1375 | A Cde/cde $M^+N^-$ | 4 | 1 | 1 | Female | 5 | O Cde/cde $M^+N^+$ | 18 | At 14 days 100% alive, 18 days epithelial loss complete |
| | | | | | 6 | 2 | 2 | Female | 2½ | AB Cde/cde $M^+N^-$ | 16 | On 15th day epithelial loss began, 25% gone |
| 2 | Male | 22 | 460 | A cde/cde $M^+N^+$ | 3 | 3 | 1 | Female | 5 | O Cde/cde $M^+N^+$ | 14 | Eleven days 80% gone, 14 days all epithelium lost |
| | | | | | 5 | 4 | 2 | Female | 2½ | AB Cde/cde $M^+N^-$ | 9 | Seven days 100% survival, 9 days epithelial loss complete |
| 3 | Female | 27 | 940 | O cdE/cde $M^+N^+$ | 6 | 5 | 2 | Female | 2½ | AB Cde/cde $M^+N^-$ | 6 | Severe infection, no graft viable on 6th day dressing |
| 4 | Male | 25 | 740 | A CDe/Cde $M^+N^+$ | 3 | 6 | 2 | Female | 2½ | AB Cde/cde $M^+N^-$ | 6 | Loss probably due to infection |
| 5 | Female | 22 | 450 | A CDe/cDE $M^+N^+$ | 14 | 7 | 3 | Male | 7 | O CDe/c-e $M^+N^+$ | 8 | Loss probably due to trauma by very active patient |
| | | | | | 20 | 8 | 2 | Female | 2½ | AB Cde/cde $M^+N^-$ | 7 | One-hundred per cent epithelium lost on 7th day dressing |
| 6 | Male | 33 | 1700 | — | 7 | 9 | 4 | Male | 20 | B CDe/cde $M^+N^+$ | 18 | Gradual loss started on 5th, with 25% survival on 10th day, & completely lost on 18th day |
| | | | | | 7 | 10 | 5 | Male | 1 | B CDe/cde $M^+N^-$ | 46 | Began to dry & shrink on 39th day, gradual loss completed on 46th day |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 7 | Female | 23 | 450 | A CDE/cde $M^+N^+$ | 8 | 11 | 4 | Male | 20 | B CDe/cde $M^+N^+$ | 4 | Good until 4th day dressing, then all lost |
| 8 | Male | 26 | 850 | — | 2 | 12 | 6 | Male | 17 | O CDe/cde $M^+N^+$ | 13 | Twelve days 100% survival, 13th day all gone |
| 9 | Female | 23 | 850 | A cDE/cde $M^+N^+$ | 4 | 13 | 5 | Male | 1 | B CDe/cde $M^+N^-$ | 23 | One hundred per cent on 20th, all epithelium lost at 23 days |
| 10 | Male | 25 | 660 | A cDe/cde $M^+N^-$ | 8 | 14 | 7 | Male | 64 | A CDe/cde $M^-N^+$ | 8 | Fifty per cent gone on 7th day dressing, all epithelium lost on 8th day |
| 11 | Male | 25 | 990 | A cDe/cde $M^+N^-$ | 8 | 15 | 7 | Male | 64 | A CDe/cde $M^-N^+$ | 8 | Fifty per cent gone on 7th day dressing, all epithelium lost on 8th day |
| | | | | | 10 | 16 | 5 | Male | 1 | B CDe/cde $M^+N^-$ | 7 | One third of epithelium gone in 6 days, 7 days all gone |
| 12 | Male | 17 | 160 | — | 5 | 17 | 5 | Male | 1 | B CDe/cde $M^+N^-$ | 4 | Rapid epithelial loss between 3rd and 4th day |

showed moderate mononuclear cellular infiltration  A nonviable biopsy was taken from the intrahepatic implant 44 days postimplantation

Fetus No 2, aged 8 weeks, was homoimplanted subcutaneously into the flank of a human recipient  At the time of presentation of this paper, the implant was surviving for 41 days but, on microscopic examination of the biopsy, there was massive mononuclear cell infiltration around the viable skin and cartilage  On the sixtieth day no fetal tissue was present, but only a marked mononuclear cell infiltration with many giant cells

Fetus No 3, aged 10 weeks, was homoimplanted into rats and rabbits  The homoimplant, when biopsied 27 days after grafting, revealed viable cartilage surrounded by a very pronounced homograft reaction

Fetus No 4, aged 11 weeks, was implanted into a male and a female human recipient as well as into adult rats and rabbits  The heteroimplants have been biopsied (22 days), and these revealed histologically viable tissues with minimal reaction around the grafts  The homoimplant biopsied after 39 days revealed viable cartilage with a marked mononuclear cell response

Tissues from fetuses No 5 and 6, after 13 weeks gestation, were both implanted into 4 humans, 2 rabbits, and 4 rats  The last biopsy carried out on homoimplanted tissue revealed viable skin and cartilage, with minimal cellular infiltration at 19 days  The heteroimplants at 21 days revealed viable cartilage with some homograft reaction

## *Conclusion*

The viability of the fetal skin grafts (17 to 33 weeks) upon application to fresh raw beds was never in doubt because, 2 to 3 days postoperatively, when the first dressing was carefully performed, they were deep pink in color, blanched on gentle pressure, and the epithelium was intact  Evaluation of viability of the tissues of fetuses (7 5 to 13 weeks) was determined by the following three criteria  first, active growth of fetal cells in tissue culture was observed in all instances when this technique was employed, second, there was a steady increase in the size of the palpable mass during the first 2 to 3 weeks after subcutaneous implantation in humans and also in animals, third, the early biopsy specimens revealed the presence of living cells on histological examination

Evidence of a typical homograft rejection response was present in biopsies of fetal skin grafts (17 to 33 weeks) applied to fresh wounds  These were taken just prior to or after loss of the epithelium  Fetal tissue homoimplants (7 5 to 13 weeks) showed a definite zone of lymphocytes and plasma cells surrounding and invading the implant between the second and third week  Biopsies taken at a later date exhibited either a more pronounced reaction or disappearance of the fetal tissues and a residual mass of granulation tissue  In general, the response of heteroimplants in the rat and rabbit to these very young fetal tissues was the same

## *Summary*

(1) It appears that, under the conditions of this experiment, fetal skin and tissue homografts evoked an active homograft rejection response

(2) All skin grafts from 17- to 33-week fetuses were rejected The average survival period was 12 6 days

(3) Biopsies of homoimplants and heteroimplants of fetal tissues (7 5 to 13 weeks) performed at this time indicate that the majority, if not all of these, will be rejected

## *References*


1 LEOPOLD, G 1881 Arch Gynäkol **18** 53
2 BIRCH-HIRSCHFELD, A & S GARTEN 1899 Beitr pathol Anat u allgem Pathol **26** 131
3 DEL CONTE, G 1907 Beitr pathol Anat u allgem Pathol **42** 193
4 MINERVINI, R 1913 Trans 17th Intern Congr Med London Surg **7**(2) 75
5 WILLIS, R A. 1935 Proc Roy Soc London **B117** 400
6 STONE, H B, J C OWINGS & G O GEY 1935 Surg Gynecol Obstet **60** 390
7 GREEN, H 1943 Cancer **3** 809
8 GAILLARD, P J 1948 Symposia Soc Exptl Biol II Growth 139
9 KOOREMAN, P J & P J GAILLARD 1950 Overg u Arch Chir Neerl **11** 326
10 HARDLER, A M & G YERGANIAN 1954 Proc Am Assoc Cancer Research **1** 18
11 STERLING, J A & R GOLDSMITH 1954 Transplantation Bull **1** 140
12 TOOLAN, H 1954 Proc Soc Exptl Biol Med **86** 607
13 GREEN, H 1943 Cancer Research **15** 170
14 ROSEN, S M & W K SILVERS 1956 Transplantation Bull **3** 5
15 MAY, R M 1957 Ann N Y Acad Sci **64**(5) 937
16 GAILLARD, P J 1954 J Natl Cancer Inst **14** 683
17 HELSINGEN, N & P HELSINGEN 1957 Transplantation Bull **4** 24
18 TOOLAN, H 1957 Transplantation Bull **4** 107
19 WOODRUFF, M F A & T M ALLEN 1953 Brit J Plastic Surg **5** 238
20 ROGERS, B O 1957 Ann N Y Acad Sci **64**(5)· 741

# EMBRYONIC PARABIOSIS AND RELATED PROBLEMS

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The study of the ontogeny of mechanisms of tissue incompatibility is rightly considered a path toward an explanation of the causes of the origin of transplantation immunity, and perhaps also as a means of successfully overcoming failures in homotransplantation of tissues The interesting results obtained by Owen[1] and by Medawar *et al*[2, 3] in dizygotic cattle twins indicated one such possibility

Embryonal parabiosis of chicks makes it possible to produce experimental conditions similar to those occurring in natural cattle parabionts Two bird fetuses are joined by their chorioallantoic membranes and, within a short period, the circulations of the two embryos are joined Parabiosis is interrupted physiologically on hatching

Using embryonal parabiosis, the hypothesis of Burnet and Fenner[4] was confirmed experimentally This hypothesis maintained that exposure of embryos to foreign antigenic substances may cause specific suppression of immune responses during postembryonic life Parabionts cannot form mutual antibodies after immunization with blood cells although, in chickens, immunization with blood cells from a randomly chosen individual practically always leads to the formation of antibody [5] It was shown later that the inability to form agglutinins is maintained even when blood chimeras no longer can be demonstrated in these animals [6]

Using embryonal parabiosis, immunological tolerance of homotransplants was induced experimentally,[7] thus confirming the results of Billingham *et al*[8] concerning acquired tolerance of homotransplants Tolerated homotransplants survive for long periods of time in parabionts However, it is not yet known why, after long periods of survival, they are sloughed Of 15 tolerated homotransplants studied over a long period in chickens, 12 survived 6 to 18 months, 2 for more than 2 years, and 1 has survived 4 years at the time of writing

Eggs from different species were also used for experimental parabiosis Parabionts between chickens and pheasants, chickens and guinea fowl, chickens and turkeys, and chickens and ducks were studied It could be shown that the degree of immunological tolerance of heterotransplants is much less than with homotransplants Heterotransplants in parabionts can survive for longer periods than in controls, but tolerance soon disappears [9] Similar results were obtained when studying immune heteroagglutinin formation In interspecific parabionts, decreased agglutinin formation was found, as distinguished from the finding in control animals, but this was less obvious than in intraspecific parabionts [10] These results indicate the significance of the quality of the antigenic stimulus when inducing immunological tolerance The degree of immunological tolerance with relation to tissue cells depends upon the taxonomic relation between the donor and the recipient

Last year we studied the immunological behavior of embryonal parabionts between the turkey and the chicken Antibody response, blood chimerism, and tolerance of skin grafts were investigated

We did not use partners from embryonal parabiosis for testing, instead, normal animals of other species were used It has been shown earlier that, in distinction to intraspecific relations, individual antigenic differences are not important in interspecific tests A chicken skin graft is tolerated by a pheasant for as long a time whether or not the donor chicken was a partner in embryonal parabiosis[9] The decrease in agglutination titers in duck parabionts with chickens is the same, whether or not they were immunized with blood from the partner or from randomly chosen birds[6] For this reason the donors were randomly selected birds of the other species

Immunization of chicken parabionts with turkey blood confirmed our previous findings[11] Natural heteroagglutinins to turkey erythrocytes were never found in any parabiont before immunization, although they can be demonstrated by using the same method in the majority of control animals Immune agglutinin formation to turkey erythrocytes was strongly depressed in parabionts and was completely absent at the beginning Only after reimmunization or transfusion of 7 5 to 10 ml of turkey blood did it appear in all parabionts studied (TABLE 1)

It was found further that turkey erythrocytes could be demonstrated in the circulation during the fifth week in 6 parabionts and, during the seventh week, in 4 of the 8 chicken parabionts studied (TABLE 2) It may be assumed that in the majority, if not all, of the chick parabionts in which turkey erythrocytes occurred over such extended periods there is interspecific blood chimerism According to the literature, the maximum life of an adult chicken red blood cell is about 5 weeks, while for ducks and pigeons it is 6 weeks (Ottesen,[12] Brace and Altland,[13] Rodnan *et al*[14]) We know of no work concerning the length of survival of turkey erythrocytes, but it may be assumed that the period will be about the same as in chickens In addition, the literature speaks of adult erythrocytes, the life of embryonal blood cells is even shorter Finally, as the animals grow, their blood volume increases This in itself would cause such a dilution of passively exchanged blood cells during parabiosis that their final amount, after 2 months, could hardly be demonstrated by the agglutination technique used

In one parabiont turkey, erythrocytes survived until the bird died during the tenth week after hatching They thus survived in the bird's circulation for 6 weeks after the skin graft had been sloughed This graft had been transplanted in the second week after hatching and was completely destroyed within 16 days In one of another set of parabionts that received a turkey skin graft at the same age, turkey erythrocytes disappeared in the fourth week after hatching, that is, at the time the graft was destroyed In the second they disappeared in the sixth week after hatching and were found for the last time 10 days after the complete destruction of the graft

In one turkey parabiont, a chicken skin graft survived for 3 months, and feathers grew on it during that period The chicken donor was brown, the

TABLE 1

IMMUNIZATION AND REIMMUNIZATION OF CHICK PARABIONTS AND CONTROLS IN THE EIGHTH AND TWELFTH WEEK OF LIFE*

| Number of animal | Natural heteroagglut. | Immunization | | | | | Reimmunization | | | | | Transfusion 5th to 6th day after transfusion |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 3d day after immunization | 6th day | 9th day | 12th day | 15th day | before reimmunization | 3d day | 6th day | 9th day | 12th day | |
| *Parabionts* | | | | | | | | | | | | |
| 904/5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0(1) |
| 3671/2 | — | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| 3677 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | → died | | |
| 3678/9 | 0 | 0 | — | 0 | 0 | 0 | 0(1) | 0(1) | 0(1) | 0(1) | 0 | 16 |
| 4239/40 | — | 0 | 0 | 0 | 0 | — | — | — | — | — | — | — |
| 4283/4 | — | 0 | 0 | 0 | 0 | — | — | — | — | — | — | — |
| *Controls* | | | | | | | | | | | | |
| 1492 | 0 | 1 | 8 | 8 | 4 | 2 | 8 | 4 | 16 | 8 | 8 | — |
| 3669 | 4 | 1 | 16 | 8 | 4 | 8 | 4 | 8 | 16 | 8 | 8 | 16 |
| 3670 | 1 | 2 | 0 | 8 | 2 | 2 | 4 | 8 | 2 | 8 | 8 | — |
| 3673 | 1 | 2 | 16 | 16 | 4 | 2 | 2 | 4 | 32 | 16 | 8 | 128 |
| 3674 | — | 0 | 8 | 8 | 4 | 16 | | — | — | — | — | — |
| 3675 | 4 | 16 | 32 | 8 | 4 | 2 | | — | — | — | — | — |
| 3676 | 0 | 0 | 0 | 0 | → died | | | | | | | |
| 3700 | — | 0 | 16 | 16 | 4 | 2 | 2 | 2 | 8 | 8 → | died | |
| 4244 | — | 1 | 2 | 2 | 1 | — | — | — | — | — | — | — |
| 4260 | — | 4 | 8 | 8 | 4 | — | | | — | — | — | — |
| 4261 | — | 16 | 32 | 4 | 2 | — | — | — | | — | — | — |
| 4264 | — | 4 | 16 | 16 | 4 | — | — | — | — | — | — | — |
| 4265 | — | 8 | 8 | 8 | 4 | — | — | — | — | — | — | — |
| 4266 | — | 0 | 0 | 0 | 0 | — | — | — | — | — | — | — |

* Immunization with 0 5 ml of citrated turkey blood, reimmunization with 1 ml of turkey blood, determination of agglutinations after ½ hour and 1 hour, transfusion of 7 5–9 5 ml of turkey blood All 3 administrations intravenous

TABLE 2

SURVIVAL OF TURKEY ERYTHROCYTES IN CHICKEN PARABIONTS AFTER HATCHING

| Number of parabionts | Time of last finding of turkey erythrocytes in the circulation (weeks) | Note |
|---|---|---|
| 3671/2 | 10 | |
| 3677 | 7 | |
| 3678/9 | 4 | |
| 3682/3 | 5 | Positive during sixth week |
| 904/5 | $\leq$8 | Negative during tenth week |
| 35/6 | 3 | |
| 37/8 | 5 | Skin transplantation |
| 52/3 | $\leq$10 | positive during eleventh week |

* Symbol $\leq$, no greater than

turkey recipient white The feathers on the heterograft were partly dark, but in parts they were white or gray Their lateral orientation showed that these also grew from the graft That this was so became apparent because the graft had been rotated by 90° In 3 other turkey parabionts, skin grafts survived longer than in controls and were destroyed in a completely different manner In parabionts, destruction was less acute and usually without the formation of crusts

In the circulation of parabiont No 12/13, in which the chicken graft survived for 3 months, no chicken erythrocytes were found at the time feathers began to grow on the graft On the other hand, parabiont No 10/11, in which the graft was destroyed relatively early, chicken erythrocytes could still be demonstrated, that is, 8 months after hatching and a number of months after the destruction of the graft It is hardly possible to deny the existence of interspecific chimerism in this case

The long survival of the chicken skin graft without erythrocyte chimerism in turkey No 12/13 might be explained in 2 ways (1) chimerism did not occur during experimental parabiosis, and (2) if it did exist, it disappeared due to some nonimmunological factors One possibility is that foreign cells were implanted in organs of embryonal blood formation only During postembryogenesis, chimerism could disappear, together with the blood-forming function of the organ

It is more difficult to explain chimerism in birds of both species after destruction of the skin graft It is highly improbable that the immunity mechanism to skin grafts is so very different from that directed against implanted blood cells Consequently, it can hardly be supposed that tolerance can be induced only toward a graft of one kind In view of the fact that the criteria we use for evaluation of the taking of skin grafts are not completely reliable, it is much more probable that evaluation of the state of the graft is inaccurate

It follows from our results that immunological tolerance between the chicken and the turkey leads in parabionts to nearly the same state as intraspecific tolerance between chicken parabionts

We now propose to mention certain problems connected with immunological

tolerance of homologous tissue The mechanism of occurrence of suppression or of the absence of formation of the abilities to respond to a foreign antibody is not yet known, just as we do not yet know the mechanisms of antibody formation Discussion of the hypotheses that are more or less founded on solid bases would exceed the limits of this report but, as far as tolerance to homologous antigens that play a part in transplantation is concerned, it is necessary to stress that the induction of immunological tolerance is still performed, as of this date, only with living cells that can be repopulated

Billingham *et al* [15] have made great progress in the analysis of the antigenic stimulus in homotransplantation They have shown that the active antigenic stimulus is present in the isolated desoxyribonucleoprotein of the nuclei V Haškova has therefore investigated the possibility of inducing tolerance to the homograft by means of isolated antigenic substances However, this investigator has not yet succeeded in eliciting the same rate of tolerance of the skin graft by means of aqueous nucleohistone extract (NHE) as reported by Crampton *et al* [17] However, NHE thus prepared is antigenically active during transplantation immunity testing in mice by means of Medawar's test [18, 19] As the question of the antigenic material qualities could be of some importance, Haškova used, for the induction of tolerance, not only chicken embryos but also newborn ducklings which, unlike chickens, have an adaptive period for the homologous antigen that lasts for several days after hatching [16] For this reason it is possible to administer a great quantity of NHE to one animal repeatedly However, the skin grafts, applied at the age of 4 weeks, did not show any signs of tolerance Either the substances used do not always contain antigens that play a part in transplantation immunity (the test for tolerance, unlike that for immunity, requires the presence of all antigens) or the antigenic substances thus prepared meet a different fate in the host organism

*References*


1 OWEN, R. D 1945 Science. 102 730
2 ANDERSEN, D, R. E BILLINGHAM, G H. LAMPKIN & P B MEDAWAR. 1951 Heredity 5 379
3 BILLINGHAM, R. E, G H LAMPKIN, P B MEDAWAR & H L. WILLIAMS 1952 Heredity 6 201
4 BURNET, F M & F FENNER 1949 The Production of Antibodies 2nd ed Macmillan Melbourne, Australia
5 HAŠEK, M 1953 Českoslov biol 2 265
6 HAŠEK, M & T HRABA 1955 Folia Biológica Prague 1 1
7 HAŠEK, M 1955 Časopis lékáru českych 94 41
8 BILLINGHAM, R. E, L BRENT & P B MEDAWAR. 1953 Nature. 172 602
9 HAŠEK, M 1954 Českoslov biol 3 327
10 HAŠEK, M 1956 Proc. Roy Soc London B146 67
11 HRABA, T 1956 Folia Biológica Prague 2 165
12 OTTESEN, J 1948 Nature 162 730
13 BRACE, K. C, P D ALTLAND 1956 Proc. Soc Exptl Biol Med 92 615
14 RODNAN, G P, F G EBOUGH, JR & M R S FOX 1957 Blood 12 355
15 BILLINGHAM, R E, L BRENT & P B MEDAWAR 1956 Nature 178 514
16 HAŠKOVA, V 1957 Českoslov biol 6 81
17 CRAMPTON, C F, R. LIPSHÜTZ & E CHARGAFF 1954 J Biol. Chem 211 125
18 MEDAWAR, P B 1944 J Anat 78 176
19 MEDAWAR, P B 1945 J Anat 79. 157

*Discussion of the Paper*

A A Lazzarini, Jr (*New York University Post Graduate Medical School, New York, N Y*) With reference to Milan Hašek's very illuminating paper, we also have been interested in studies of embryonal parabiosis in birds for some time The method used was similar to that so successfully developed by Hašek for single pairs of eggs

A group of parabiotic teams of Rhode Island Red and White Leghorn chicken eggs, each team comprising 3 to 7 eggs, were connected in alternated combinations in order to have embryos with 2 or 3 parabiotic mates at the same time and in which a successful exchange of skin transplants could be made We found, first, that when parabiotic teams of either Rhode Island Red or White Leghorn chicken eggs were prepared with mates of the same strain, the survival rate was 20 to 35 per cent higher than when an interstrain combination was used Second, we found that the survival rate after hatching was higher when the eggs had been incubated for 8 to 12 days at the time of parabiosis In young embryos the scarce development of chorioallantoid vessels made it necessary to place the window closer to the air chamber

# HOMOTRANSPLANTATION OF DEVELOPING TOOTH BUDS IN THE RAT*

*By* Harry H Shapiro and Dorothy D Johnson
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## *Introduction*

This experimental investigation was undertaken to determine the capacity of a developing tooth bud to differentiate normally when transplanted into an alveolus of a young rat and to study the results of such transplantation histologically

Roentgenographic studies of tooth-bud transplantation into the alveolar sockets of kittens have been reported previously by one of us (H.H S )[1,2] Various locations in the body have served as host sites for investigators working with transplanted developing teeth of lower animals [3-7] Important *in vitro* studies[8 12] deal with various aspects of the transplantation of teeth or of tooth buds Only a few other investigators[13 14] have selected the alveolar socket as a site for the study of *in vivo* transplantation of teeth

A recent publication dealing with the transplantation, replantation, and surgical positioning of teeth[15] indicates increased interest in clinical aspects of these studies in man It is our feeling that, before indulging in such procedures in man, a more complete awareness of the effects of transplantation upon the various components of the developing tooth bud is essential, these components include the enamel organ (dental organ), the odontoblasts (dentin-forming cells), the ameloblasts (enamel-forming cells), the dentin, enamel, and connective tissue of the dental pulp and the surrounding area, the vascular supply, the periodontal membrane, the cementum, and the supporting alveolar bone

A study such as this involves the differentiation and growth of a relatively embryonic group of tissues that have been transplanted to sites other than their native ones, but that have, nevertheless, relatively normal environments Because additional knowledge of the effects of transplantation of developing tooth buds on the components of the teeth and their supporting tissues remains a prime requirement, especially since such studies are not possible in the transplantation of teeth as now being practiced in man, we are confining our investigation to *in vivo* experiments related to these basic considerations

This report is limited to a study of the developing tooth bud in the early phases following transplantation into the mandible of a host animal We are currently proceeding with an investigation of additional aspects of the problem, such as the effects of transplantation upon the transplant in the later phases of development and the process of eruption

* The work reported in this paper was supported in part by Grant DA 49 007 MD 718 from the Department of the Army, Washington, D C

*Methods, Materials, and Techniques*

Young rats aged 24 to 28 days are used as host animals, since rats at this age can be weaned and, after operation, continue to gain weight steadily on an ordinary adult rat diet Also at this age the third molar tooth bud has not as yet erupted into the oral cavity, and can therefore be removed by a lateral approach without injury to the oral plate of alveolar bone or the oral mucosa

Still younger rats aged 5 to 10 days served as donor animals Tooth buds, of the first molar, even from young rats 3 to 4 days old, are too large to transplant into third molar sockets of host animals, due to the inherent difference in the normal size of the molar teeth in the rat

We removed buds of the second molar from donor animals 5 to 10 days of age and transplanted them into the third molar sockets of host animals Buds of the third molar were removed from 15- to 18-day-old donor rats and were similarly transplanted

We employed pentobarbital sodium (Nembutal) at a dosage of 8 mg /100 gm of body weight, as the anesthetic of choice Given intraperitoneally, it is a convenient and satisfactory anesthetic One injection provides adequate anesthesia for the duration of the entire operation, the animal becomes quite active within one half hour after the surgical procedure is completed

We have also developed a satisfactory surgical technique for the removal of the third molar tooth bud from the host animal After removing the hair from the lateral surface of the face, an incision is made through the skin, exposing the underlying masseter muscle The muscle is then separated by blunt dissection and retracted, thus exposing the outer surface of the posterior surface of the mandible A circular piece of bone, directly above the developing third molar tooth bud of the host animal, is then removed, employing an electrically driven engine and a special small trephine, the section of bone, about 4 mm in diameter, is set aside for later replacement The tooth bud is then carefully removed with a small spoon excavator The donor animal is operated upon similarly, and the second molar tooth bud or, in other cases, that of the third molar, is removed and transplanted into the socket established in the host

Great care must be exercised in removing and transferring the developing tooth bud from the jaw of the donor in order to preserve the entire dental follicle and its contents without injury, these are extremely fragile in the early stages of development

One simple suture (Nylon thread) in the muscle and two in the skin are sufficient to close the area and to protect the bone during the healing phase With relatively clean, careful technique, infections in the operated area are rare

To date we have operated upon 132 experimental animals Thirty-one of these were used for exposure of the third molar area and removal of the tooth bud, without transplantation of another tooth bud into the socket thus formed These young rats were sacrificed at intervals from 7 days to 28 days after op-

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FIGURE 1 (a) Photomicrograph of a section of a normal second molar tooth bud removed from the mandible of a 7-day-old rat and fixed for histological examination The components of the enamel organ, ameloblasts, odontoblasts, forming dentin, dental pulp, and follicular sac are visible ×42 (b) Photomicrograph of a transplanted second molar tooth bud 7 days after transplantation The transplant is becoming established and is well oriented in the jaw of the host animal Hematoxylin and eosin ×74

eration. The mandibles, both operated and control sides, were decalcified, paraffin-embedded, sectioned, and stained. Histological examination of sections of the mandible from which the third molar tooth bud was removed showed that removal is complete, leaving a clean socket, with no traces of tooth bud to regenerate, that growth of new vascular connective tissue into the empty socket begins very soon after operation, and that new growth of bone into the area of bone removal is rapid, provided there has been no undue trauma or infection. In such instances, the gap is filled by rather dense, scar-like connective tissue.

In the remaining 101 experimental animals, developing molar tooth buds from younger donor rats were transplanted into the host mandible immediately following the removal of the third molar tooth bud. These animals were sacrificed at intervals of from 1 week to 2 months after operation.

## *Observations*

FIGURE 1*a* shows a section of a second molar tooth bud that was fixed for histological examination immediately upon its removal from the mandible of a 7-day-old donor animal. This is representative of the appearance of a normally developing second molar tooth bud at an age comparable to those used for transplantation into the mandibles of host animals. The various components of the enamel organ, the partially developed crown and cusps of the tooth, the ameloblasts, the odontoblasts, the forming dentin, vessels in the dental pulp, and the surrounding follicular sac can all be identified.

FIGURE 1*b* shows a section of the area of the mandible of a host rat into which a developing second molar tooth bud had been transplanted 7 days prior to the sacrifice of the animal. It can be seen that (1) the transplant is becoming established in the alveolus of the host, (2) it is well oriented in the bone, as evidenced by its relation to the oral epithelium above and to the incisor tooth below, (3) the dental pulp appears to be in good condition and vessels in the pulp are evident, (4) the alveolar bone is developing around the transplant, but not between the transplant and the oral epithelium, and (5) only a slight amount of new dentin has been deposited at this time, due probably to the period of adjustment of the transplant to its new environment.

In order to study possible effects of positioning upon the transplanted developing tooth bud, we deliberately placed some of the transplants upside down in the mandible of the host. FIGURE 2 shows a seven-day-postoperative second molar tooth bud transplant, intentionally placed in such a position. A number of such abnormally placed transplants have become established and have developed, but have not shown indications of eruption into the oral cavity; if permitted to grow they would presumably remain impacted in the host mandible. The transplant, although inverted, is becoming established as do the normally positioned transplants. The pulp is in good condition, with blood vessels present. The layer of dentin-forming cells (odontoblasts) can be seen lining the outer aspects of the pulp. Some new dentin has formed; hypertrophy of the dentin can be identified toward the neck of the crown of the tooth, probably due to some injury of the surrounding follicular




FIGURE 3 (a) Photomicrograph of a developing second molar 23 days after transplantation The transplant is well established and is beginning to erupt into the oral cavity See text for details Hematoxylin and eosin ×36 (b) Higher magnification of the same section, showing cusp of the transplant erupting into the oral cavity and the epithelial attachment on the left side of the cusp Hematoxylin and eosin ×74



FIGURE 2 Developing second molar tooth bud in the mandible of the host animal 7 days after transplantation The transplant, although placed in an upside-down relation in the jaw, is becoming established Such abnormally placed transplants show no indication of eruption Hematoxylin and eosin X74

sac at the time of transplantation The central area close to the oral epithelium demarks the separation of the developing roots of the tooth bud, a thickening of the epithelium is seen above this The space below the transplant is an area of inflammation

FIGURES 3*a* and *b* show a section of a 23-day postoperative transplant of the second molar The transplant is well established and the orientation in the jaw is good, although the tooth bud has been placed at a slightly tilted angle A considerable amount of new dentin has been deposited since the time of transplantation, the amount of dentin can be estimated by the sharp line of demarcation between the older and more recently deposited dentin The dentin appears normal and well organized, except where the ameloblasts or enamel over it have been injured, in such areas the dentin takes on an abnormal appearance The developing tooth is beginning to erupt into the oral cavity The epithelial attachment (that part of the reduced enamel organ that will attach the crown portion of the erupted tooth to the gingival tissues) can be seen clearly on the upper left side of the cusp of the crown That this tissue appears to react normally during the eruption process is particularly gratifying in view of the ever-present danger of injury to the follicular sac and the enamel organ during the removal and transplantation of the developing tooth bud The root of the transplant has grown considerably

(3) As the transplant grows, the cusps elongate, with the formation of new dentin, and the roots begin to develop

(4) Under favorable circumstances, uninjured, well-oriented transplants become established in the mandible of the host, and the growing transplants begin to erupt into the oral cavity

(5) There still remains much to be studied in these processes with regard to the role played by such tissues as the periodontal membrane, the cementum, and the loose connective tissue of the oral mucosa

(6) Investigation of the later phases of development of the transplant and the process of eruption are currently under investigation

*References*


1 SHAPIRO, H H & B L MACLEAN 1942 Anat Record **82** 489
2 SHAPIRO, H H & B L MACLEAN 1945 J Dental Research **24** 93
3 HUGGINS, C B , H R. MCCARROLL & A A DAHLBERG 1934 J Exptl Med **60: 199.**
4 SUTRO, C J & L POMERANTZ 1939 A. M A Arch Pathol **28** 199
5 HAHN, E 1941 J Dental Research **20** 5
6 VILLAFANE, I Z & J C LASCANO GONZALEZ 1942 Rev asoc méd arg **56**· 71
7 FLEMING, S F 1952 J Dental Research **31** 166
8 YUMIKARA, S 1925 Arch pathol Anat u Physiol Virchow's **254** 17
9 GLASSTONE, S 1936 J Anat **70** 260
10 LOSEE, F L 1943 U S Naval Bull **41** 758
11 LEFKOWITZ, W & P SWAYNE 1955 J Dental Research **34** 706
12 SZABO, G 1954 J Anat **88** 31
13 LAPSCHINSKY, A & A. A MALINOVSKY 1941 Lancet **241** 78
14 AGNEW, R G & C FONG 1955 J Dental Research **34** 669
15 FLEMING, S F 1956 Oral Surg Oral Med Oral Pathol **9** 3



FIGURE 4 Photomicrograph of developing third molar tooth bud transplant in host mandible 28 days after transplantation The transplant is well established and well oriented A great deal of new dentin has been deposited Periodic acid-Schiff ×42

FIGURE 4 shows a section of a 28-day postoperative third-molar tooth bud transplant This tooth bud was transplanted into the alveolus of a slightly younger host animal, aged 24 rather than 28 days at the time of transplantation At this earlier period the covering bone between the developing tooth bud and the oral epithelium has not as yet been resorbed It is known that the developing tooth bud normally influences the resorption of the covering alveolar bone The transplant is well established and well oriented Formation of the root is beginning The inherent morphology of the third molar tooth bud differs from that of the second molar, the second molar teeth do not have the same number of cusps or roots, the appearance of the transplant is therefore different from second-molar transplants The pulp and odontoblasts are in very good condition A periodic acid-Schiff staining technique was used in this case, which explains the heavy staining of the dentin A great deal of new dentin has been deposited and can be observed, especially on the left side of the transplant

## *Conclusions*

(1) The alveolar socket formed by the removal of a third molar tooth bud in the rat is an ideal location for transplanted younger molar tooth buds

(2) Establishment, vascularization, and growth of the transplanted tooth bud are assured, regardless of the orientation of the transplant

(3) As the transplant grows, the cusps elongate, with the formation of new dentin, and the roots begin to develop

(4) Under favorable circumstances, uninjured, well-oriented transplants become established in the mandible of the host, and the growing transplants begin to erupt into the oral cavity

(5) There still remains much to be studied in these processes with regard to the role played by such tissues as the periodontal membrane, the cementum, and the loose connective tissue of the oral mucosa

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1 SHAPIRO, H H & B L MACLEAN 1942 Anat Record **82** 489
2 SHAPIRO, H H & B L MACLEAN 1945 J Dental Research **24** 93
3 HUGGINS, C B, H R MCCARROLL & A A DAHLBERG 1934 J Exptl Med **60** 199.
4 SUTRO, C J & L POMERANTZ 1939 A M A. Arch Pathol **28** 199
5 HAHN, E 1941 J Dental Research **20** 5
6 VILLAFANE, I Z & J C LASCANO GONZALEZ 1942 Rev asoc méd arg **56** 71
7 FLEMING, S F 1952 J Dental Research. **31** 166
8 YUMIKARA, S 1925 Arch pathol Anat u Physiol Virchow's **254** 17
9 GLASSTONE, S 1936 J Anat **70** 260
10 LOSEE, F L 1943 U S Naval Bull **41** 758
11 LEFKOWITZ, W & P SWAYNE 1955 J Dental Research **34** 706
12 SZABO, G 1954 J Anat **88** 31
13 LAPSCHINSKY, A & A A MALINOVSKY 1941 Lancet **241** 78
14 AGNEW, R G & C FONG 1955 J Dental Research **34** 669
15 FLEMING, S F 1956 Oral Surg Oral Med Oral Pathol **9** 3

# BEHAVIOR OF SKIN GRAFTS EXCHANGED BETWEEN PARENTS AND OFFSPRING*

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In a previous paper I reported the successful transfer of skin from a mother to a 3-month-old male infant that had received, at the age of 3 weeks, an intramuscular injection of 2 ml of maternal blood [1] This boy has been followed with biopsy and electrophoresis studies and had not rejected the skin graft of the mother by 240 days At this time the graft was removed and microscopic examination demonstrated normal epidermis, but there was a very dense infiltration of lymphocytes within the dermis and an absence of hairs and glands The adjacent host skin was entirely free of this lymphocytic infiltration Epidermal cells in the homograft showed the sex chromatin that is characteristic of female skin [2]

The injection procedure was followed on the assumption that pretreatment with cells from a prospective skin donor might create a state of tolerance in young infants similar to that observed in the experimental work of Medawar *et al* [3] These investigators injected fetal mice or chicks with living homogenous cells and thereby created tolerance of skin grafts from donors that had provided the inoculum Using another species (rats) Woodruff[4] injected the young on the day of birth with splenic breis and noted that these newborn rats were tolerant in later life to skin grafts from the rat that provided the injection Applying this principle to humans Woodruff,[5] in two cases, injected living leukocytes from fathers into the thigh muscles of their respective infants 48 and 3 hours after birth When the infants were 6 months old each received a split graft from its father Both grafts were initial takes and persisted until 4 weeks, but thereafter decreased considerably in size

Thirteen additional infants of both sexes have been injected intramuscularly by our group[6] with 4 ml of whole blood taken from parents or grandparents with compatible blood groups (blood groups were determined only as to O, A, B, AB, and Rh factors) The age of the infants at the time of injection varied from 3 to 28 days, and the time interval between injection and subsequent skin transplantation ranged from 40 to 75 days Full-thickness skin grafts were interchanged between parents and infants in 11 cases In the remaining 2 cases the transplantation was only from parents to infants Three exchanges were with fathers, one with a grandfather, and the remaining 9 with mothers (FIGURE 1)

Dark discoloration or mottling of the graft was considered the end point of survival Two grafts from infants to mothers developed superficial blebs in the central quarter but both healed, apparently by proliferation of graft epithelium, these were evaluated as successful and are still in place 97 and 99 days, respectively, following transplantation Prolonged survival of the grafts

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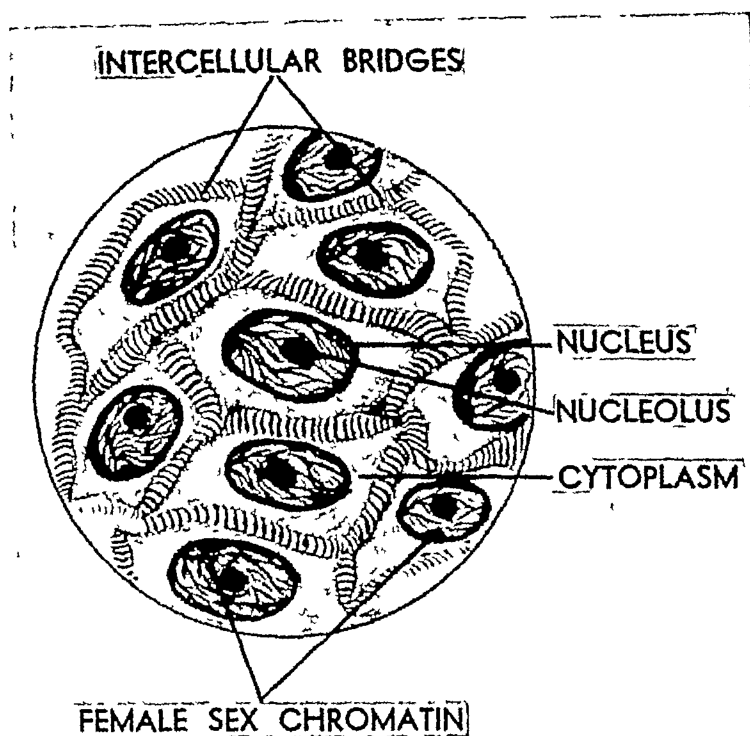



FIGURE 1 Drawing of epidermal cells of human female skin Note the characteristic sex chromatin in contact with the nuclear membrane ×1000

on infants or parents did not appear to be related to the sex of the child or its age at the time of blood injection or to the time interval between blood injection and skin exchange, or to be dependent on any fixed pattern of blood groups We found, however, that all prolonged survivals occurred when the grafts were exchanged between mother and infant Long-surviving grafts from mother to infant have persisted for 213 days A rather surprising finding was the still longer survival time of the skin of the child behind the ear of the mother, these homografts have remained for as long as 15 months

The grafts exchanged between fathers and infants and one grandfather and infant were all rejected both ways The longest survival of male parental or grandparental skin on infants was 14 days The longest survival of infant skin on father or grandfather was 20 days The survival times of grafts interchanged between mother and child had no apparent correlation On occasion a mother retained the skin of her child long after the child had rejected the skin of his mother, and vice versa The gamma-globulin levels of the infants varied, but long-surviving skin homografts occurred in children with levels of 7, 15 2, and 8 3 per cent of the total proteins as determined by electrophoresis

A control series of 33 skin interchanges between parents and infants or parents and older children with compatible blood groups, in which the offspring did not receive a blood injection from the parent, has yielded some interesting findings [6] A survey demonstrated no prolonged survival when the exchange was made between father and male or female infant Prolonged survival (up

to 250 days) has been obtained when the exchange was made between mother and male or female infant In 2 cases exchanges of skin were made between mothers and their 7- and 12-year-old male-children These homotransplants have been retained by both pairs for 78 and 253 days, respectively

Biopsy study of the homograft from mother to 12-year-old boy 3 months after transplantation demonstrated the sex chromatin in the epidermal cells that is characteristic of female skin [2] Biopsy of the boy's skin graft on the mother showed an absence of female sex chromatin The transplants, however, did not appear like autografts The epidermal layer was normal, but there was an absence of hairs and glands, and the dermis was infiltrated by a dense collection of lymphocytes Small blood vessels in the dermis contained blood cells of normal appearance

### *Conclusion*

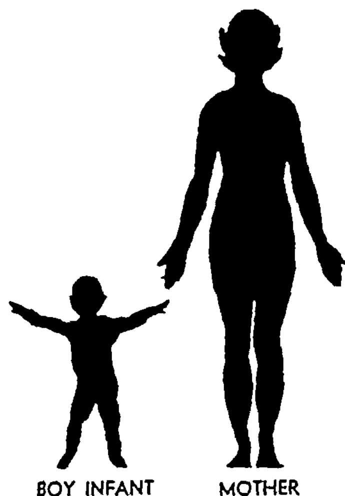
(1) The longer survival of skin homografts from mother to child than from father to child (FIGURE 2) suggests that tolerance between mother and child may occur because of fetal exposure to maternal substances (antigens?) This might lead to "acquired tolerance" in the sense of Billingham *et al* [7] The early rejection of grafts from mother to child possibly may be explained by a failure of fetal exchange to take place, for reasons unknown It appears evident from these experiments that the prolonged survival of skin grafts exchanged between mother and infant or child was not due to the blood injection, which may have been administered to the infant during a "null period" when exposure to an antigenic stimulus has no appreciable effect [7] The extremely long survival of the child's skin on the mother is difficult to explain A possible reason for the relative tolerance to father's skin (6 weeks) reported by Woodruff[6] may be the fact that he injected younger infants (48 and 3 hours old) with a much larger amount of leukocytes However, there may be some dangers associated with the injection of large numbers of leukocytes in newborn infants, and I agree with Woodruff that this should be clarified by further experimental work

(2) The prolonged survival time of skin grafts from mother to child suggests the possible clinical value of blood transfusions from mother to child rather than from fathers or unrelated donors Our preliminary findings also suggest the advisability of using mothers as donors of skin homografts for severely burned children

(3) Sex chromatin study of the epidermal cells in skin homotransplants offers a positive method for determining the survival of the graft when the exchange is made between male and female

(4) A child who tolerates his mother's skin may also tolerate other tissues from his mother, such as kidney and endocrine glands Routine skin exchanges may prove to be useful tests for tolerance to other tissues from mother to child and vice versa

(5) The long survival of skin grafts exchanged between mothers and 7- and 12-year-old children indicates that tolerance persists as the infants grow older




INJECTED INFANTS — 11 SKIN EXCHANGES

SURVIVAL — SKIN ON 2 MOTHER AND 2 INFANT PAIRS
SKIN ON 2 INFANTS
SKIN ON 2 MOTHERS

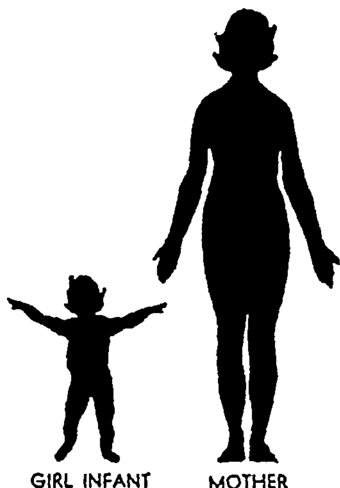
REJECTION — SKIN ON 5 MOTHER AND 5 INFANT PAIRS

UNINJECTED INFANTS — 17 SKIN EXCHANGES

SURVIVAL — SKIN ON 1 MOTHER AND 1 INFANT PAIR
SKIN ON 2 MOTHER AND 2 CHILD PAIRS
SKIN ON 3 INFANTS
SKIN ON 2 MOTHERS
SKIN ON 1 CHILD

REJECTION — SKIN ON 6 MOTHER AND 6 INFANT PAIRS
SKIN ON 2 MOTHER AND 2 CHILD PAIRS

NO APPARENT RELATIONSHIP REGARDING BLOOD GROUPS, A. B O Rh and GAMMA GLOBULIN LEVELS




INJECTED INFANTS — 2 SKIN EXCHANGES

SURVIVAL — SKIN ON 1 INFANT

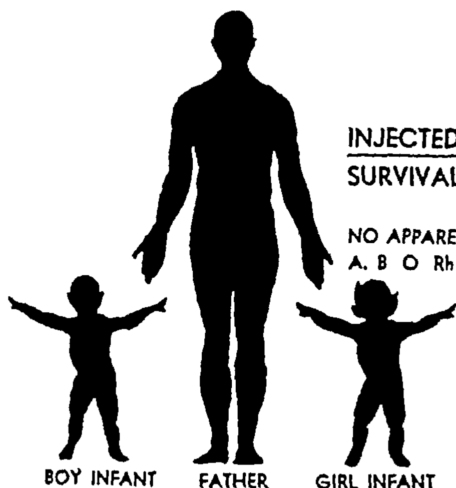
REJECTION SKIN ON 1 MOTHER AND 1 INFANT PAIR

UNINJECTED — 10 SKIN EXCHANGES

SURVIVAL — SKIN ON 3 INFANTS
SKIN ON 1 MOTHER
— SKIN ON 1 MOTHER AND 1 INFANT PAIR

REJECTION — 5 MOTHER AND 5 INFANT PAIRS

NO APPARENT RELATIONSHIP REGARDING BLOOD GROUPS, A. B O Rh and GAMMA GLOBULIN LEVELS




12 SKIN EXCHANGES

INJECTED AND UNINJECTED INFANTS —

SURVIVAL — NONE BY INFANTS AND FATHERS REGARDLESS OF BLOOD INJECTION LONGEST SURVIVAL 20 DAYS

NO APPARENT RELATIONSHIP REGARDING BLOOD GROUPS, A. B O Rh and GAMMA GLOBULIN LEVELS

FIGURE 2

### *References*

1 PEER, L A. 1956 Long survival time of skin graft from mother to male child Plastic Reconstr Surg 18 169
2 BARR, M L & E G BERTRAM 1949 A morphological distinction between neutrones of male and female, and the behavior of the nuclear satellite during accelerated nucleoprotein synthesis Nature 163 676
3 MEDAWAR, P B, R E BILLINGHAM & E M SPARROW 1953 Actively acquired tolerance Transplantation Bull 1 22
4 WOODRUFF, M F A. 1956 Post partum induction of tolerance in homologous skin in rats Transplantation Bull 3 68
5 WOODRUFF, M F A. 1957 Can tolerance to homologous skin be induced in the human infant at birth? Transplantation Bull 4 26
6 PEER, L A 1957 Behavior of skin homografts interchanged between parents and infants Transplantation Bull 4 109
7 BILLINGHAM, R. E, L BRENT & P B MEDAWAR 1956 Quantitative studies on tissue transplantation immunity Actively acquired tolerance Phil Trans Roy Soc London B239 357

### *Discussion of the Paper*

BLAIR O ROGERS (*Institute of Reconstructive Plastic Surgery, New York University-Bellevue Medical Center, and the Department of Surgery, New York University College of Medicine, New York, N Y*) Work almost completed at our research laboratories,* in which reciprocal skin grafts have been made between CFN rat brothers, sisters, parents, and grandparents, has yielded results almost identical to those reported by Peer in the human For example, we have found that the skin of female rats survives significantly longer when transplanted to the male or female offspring of the animal than does the skin of male rats when transplanted to their male or female offspring In addition, these studies appear to confirm earlier observations on humans[1] in which it was demonstrated that skin homografts obtained from a closely related human donor (for example, a brother or a fraternal twin) have temporary survival times on the human recipient that, under control conditions, are significantly longer than those of grafts from unrelated donors

I hope that the findings of Peer, as well as our own, will help geneticists as well as clinicians to establish some practical law of genetic relationship that might have direct relevance to the choice of "ideal" donor material in any future problems involved in human homotransplantation These findings already suggest that, if skin grafting is indicated for a severely burned patient, the donor material should preferably be taken from closely related members of the patient's family and, most desirably, from a mother, a brother, or a sister If followed, this practice should help to guarantee a significantly longer temporary survival time of homografts than would be the case if the grafts were taken from unrelated donors

We must remember also that Schoene[2, 3] and Loeb[4] had suggested the existence of a possible genetic gradation in transplantation that has now been demonstrated by Peer and by our group Schoene, for example, obtained his most satisfactory results in rats when transplanting between brothers and sisters In random syngenesiotransplantation between members of noninbred families

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of guinea pigs, Loeb obtained the best survival times in brother-to-brother (or sister) transplantations, whereas there was little significant difference in the slightly shorter survival time when tissues were reciprocally transplanted from parents to children or from children to parents

*References*


1 ROGERS, B O 1957 Ann N Y Acad Sci 64(5) 741
2 SCHOENE, G 1912 Die heteroplastische und homoplastische Transplantationen Springer Berlin, Germany
3 SCHOENE, G 1916 Bruns' Beitr klin Chir 99 233
4 LOEB, L 1945 The Biological Basis of Individuality Thomas Springfield, Ill


*Discussion of the Paper*

ALEXANDER WOLSKY (*Biological Laboratory, Fordham University, New York, N Y*) In our laboratory Donald Pizzarello is making skin homotransplantations on the American salamander *Triturus viridescens* His findings appear to be the same as those of Peer on humans and of Eichwald, on rodents, namely, that females are superior to males both as donors and as hosts to their own kind This may have some bearing on the hypothesis that pregnancy may be a cause of this quality of females This possibility must be ruled out in salamanders, as no pregnancy exists in these animals, their eggs are deposited in the water before they are fertilized and develop outside of the maternal body

# COMPARATIVE IMMUNOLOGY AND THE PHYLOGENY OF HOMOTRANSPLANTATION*

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## *Introduction*

To date, transplanting skin and kidneys[1] in identical twins, transplanting skin in the hypogammaglobulinemic human,[2] and occasional successes in functioning endocrine homografts[3] have been the only examples of permanent transplant survivals in man.

In invertebrates and, in some specialized circumstances, in vertebrates, homotransplantation results have not been as uniformly discouraging as they have been in man. For this reason the clinician's hopes that eventually "spare parts" may be used in human medicine have been raised.[4] Successful homotransplantation in vertebrates seems to depend on some degree of immaturity[5,6] or on alterations in the immune mechanisms of the host[7,8] (TABLE 1); in warm-blooded vertebrates "tolerance" to homografts is such an alteration.[9,11] It has been studied in both spontaneous[12,13] and experimentally produced chimeras.[9-11,14,15] Immological blockade is another of these phenomena. It was first described by Felton[16] for pneumococcus infections, by Chase[16a] for drug allergy, and since has been extended to the field of homografting[17] and cancer cell survival.[18,19] Both of these processes can be reversed: tolerance by implants of reticuloendothelial cells,[11] and immunological blockade by various accessory antigenic stimulants.[16,18] These observations, nevertheless, have stimulated a wide search among zoological phenomena for other means of controlling immunological rejection of homografts.

The purpose of this presentation is to correlate comparative phylogenetic data on plasma protein patterns and the function of the reticuloendothelial system with successful homotransplantation in various animal species.

*Comparative immunology.* Since 1884, when Metchnikoff described phagocytosis in *Daphnia*,[20] host-protective responses have been thought to be both cellular and humoral in nature. Although these two systems are present in invertebrates,[21,22] as well as in vertebrates[23] (TABLE 2), their relative importance in the survival of species varies greatly.

In lower animals,[24,25] as well as in man,[23] phagocytosis by polymorphonuclear cells and by the more slowly mobilized mononuclear cells, or so-called "lymphocytes," of invertebrates is a major defensive system for disposing of saprophytes.

In invertebrates, the phagocytic mononuclear cells are important in the

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TABLE 1

HOMOGRAFT SURVIVAL IN MAMMALS

*Embryonic conditioning*
- Spontaneous chimera
- Embryo grafting
- Experimental tolerance

*Early life grafting*
- Homografting day-old chicks
- Homografting day-old rats

*Suppression of humoral response*
- Hypogammaglobulinemia in man
- Immunological blockade-specific antigens
- Serial transplantation
- Intraocular and intracerebral transplantation
- Cortisone treatment
- Antimetabolite drugs
- Chronic uremia
- Massive X-ray exposure

TABLE 2

COMPARATIVE IMMUNOLOGY RETICULOENDOTHELIAL SYSTEMS

| | PMN* | Phagocytic monocyte | Nonphagocytic lymphocytes | Plasma cell | Acquired immunity | Natural immunity |
|---|---|---|---|---|---|---|
| *Invertebrates* | + | + | 0 | 0 | Partly specific | Variable |
| *Vertebrates* | | | | | | |
| Embryos | Early | Early | Late | Late | Late | Weak |
| Adults | + | + | + | + | Specific | Present |

* Polymorphonuclear neutrophil leukocytes

segregation phenomenon,[24,26,27] which leads to granuloma formation in somewhat the same manner in which this occurs in vertebrates[28-31] Invertebrates, however, do not possess cells corresponding to nonphagocytic lymphocytes and plasma cells of the type seen in various vertebrates[32] In these respects the development of mammalian embryonic reticuloendothelial systems recapitulates phylogeny[32,33]

*Humoral immunity* TABLE 3 gives an outline of the types of humoral immunity that have been described in various animals

Acquired immunity in invertebrates is passively transferable,[34,35] either with cells or with serum, and is relatively nonspecific[36] It appears in 12 to 14 hours[37] and usually wanes in a few days

In contrast, acquired immunity in vertebrates appears less rapidly and is more specific Humoral antibody formation in poikilothermic vertebrates can be summarized by saying that with good antigenic stimuli, applied over an adequate time, specific humoral antibodies usually can be demonstrated in adult animals[33] Natural isoagglutinins are absent in fish,[33,39] frogs,[40] snakes,[41] and alligators,[42] but are present in some turtles[43] Antitoxic antibodies may not arise in amphibia or in some species of turtles, snakes, and crocodiles[44] Anaphylaxis has been demonstrated in fish,[45] in some turtles[46] and in salamanders[47]

TABLE 3
COMPARATIVE IMMUNOLOGY HUMORAL ANTIBODIES

| | Antibodies* | Anaphylaxis | Isoagglutinins | Natural immunity |
|---|---|---|---|---|
| *Invertebrates* | | | | |
| Earthworms | — | ?+ | | Variable |
| Crabs | ± | 0 | | Variable |
| Insects | ± | 0 | | Variable |
| *Vertebrates* | | | | |
| Fish | | 0 | 0 | Present |
| 16° C | ± | | | |
| 28° C | + | | | |
| Amphibia | | + | | Present |
| 10° C | ± | | 0 | |
| 20° C | + | | 0 | |
| Reptilia | ± | + | ± | Present |
| Birds | + | + | | Present |
| Mammalia | + | + | ± | Present |

* Agglutinins, lysins, and antitoxins

These variable results in the cold-blooded vertebrates may have something to do with the temperature of the animal during immunization, since students who have worked with fish[48] and frogs[49] agree that raising the temperature of the environment accelerates antibody formation

*Other reticuloendothelial cells* Vertebrates, unlike invertebrates, possess a nonphagocytic lymphocyte and a plasma-cell system [32] In contrast to poikilothermic species that produce antibodies more slowly, birds,[50, 51] like other warm-blooded animals, have rapid immune responses to antigenic stimuli Granuloma formation and hypersensitivity have been described in fish[37, 52] and various mammals[28, 29, 53, 54] in which the lymphoid system has been held responsible for delayed-type bacterial allergy [55]

From a comparative immunological viewpoint it may be said that, in invertebrates, prolific reproduction and a short life span, coupled with useful mutations in natural immunity,[56] take the place of antibodies made by the nonphagocytic lymphoid cell[57, 58] and plasma-cell systems[59, 60] of higher animals in the preservation of the species against annihilation by infections

*Serum proteins in different animal species* The recent introduction of paper strip electrophoretic methods[61] for the study of serum proteins has made it practicable to examine the blood of a wide variety of smaller animals

TABLE 4 gives the total serum protein and electrophoretic values on several animal types We found, as have others who have studied amphibia,[62] that there are much greater individual variations in the total protein values than are seen in healthy humans

FIGURE 1 shows representative data on different protein peaks noted by electrophoretic study Their position on the paper strip, which has been labeled arbitrarily, suggests that proteins with mobilities similar to those of gamma-globulins are essentially absent in invertebrates, but are present in the various vertebrates that have been studied

TABLE 4

COMPARATIVE VALUES OF SERUM ELECTROPHORETIC COMPONENTS

The average serum total protein values on groups of animals of different species are given Determinations were done by the method of Nessler [86] The values for different serum proteins are calculated from the percentage of protein measured by paper electrophoresis, using a Spinco machine[57] with a B-3 cam, Bromphenol blue dye, and a blue Corning filter A barbiturate buffer at *p*H 8 6 with an ionic strength of 0 075 was used

The gamma-globulin peaks represent significant peaks in the vertebrates The values for crab sera may be technical residues at the point of application It will be noted that an alpha-globulin peak is a large part of the total protein of crab serum, this is probably the oxygen-carrying hemocyanin protein of their blood It also will be noted that salmon serum has a high alpha-globulin peak These animals were upstream salmon on the way to spawning Their serum cholesterol values ranged between 400 and 1200 mg per cent The frogs, all males caught in their fall migration, had been in hibernation for several months at the time they were studied The salamanders were studied at the time of spawning As indicated in the graphic samples of the electrophoretic peaks, there was a difference between sexes in serum protein values in salamanders that was not seen in the other species The considerable variation in the concentration of serum albumin, shown only as an average, probably reflects different periods of starvation characteristic of migrating fish and amphibia

| Species | No | Total protein | $\gamma$ | Values in gm % | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | $\beta_2$ | $\beta_1$ | $\alpha_2$ | $\alpha_1$ | Albumin |
| Crab | 19 | 6 01 | 22 | | | | 2 30 | 2 67 |
| Salmon | 5 | 7 57 | 78 | 1 29 | 55 | 1 22 | 1 46 | 2 42 |
| Salamander | 22 | 3 53 | 35 | 1 23 | | 66 | 70 | 50 |
| Frog | 18 | 2 84 | 45 | 70 | | 77 | | 93 |
| Rat | 19 | 5 61 | 52 | 1 16 | 35 | 29 | 73 | 2 84 |
| Guinea pig | 18 | 6 43 | 36 | 84 | | 1 39 | | 3 86 |
| Man | 86 | 7 67 | 1 18 | 1 00 | | 76 | 26 | 4 45 |

## *Transplantation in Animals*

There are numerous zoological reports (TABLE 5) of the use of heterotransplantations[63] and homotransplantations as a means of studying tissue regeneration,[64, 65] invertebrate endocrinology,[66, 67] and vertebrate embryology [68] Perhaps the richest literature on homotransplantation is that on limb bud studies,[69] neuroanatomy,[70] and endocrine transplants in embryo and larval amphibia [71]

Many factors promote or suppress homograft success in lower animals The relatively greater innate capacity of invertebrate tissues to regenerate is one factor [72] Some grafts, however, may regress to relatively undifferentiated tissue before returning to adult tissue forms [73] The relation of the graft to other tissues and to a proper nerve supply is still another contributing factor [68, 69] Even with these built-in aids, adult tissue grafts[71] and various forms of parabiosis are well tolerated in invertebrates[64, 66] and embryonic amphibia [71, 72]

Endocrine homografts have been relatively successful in invertebrates[67] as well as in vertebrates [72, 73] Growth stimulation by trophic effects in amphibia is well illustrated by the fact that placing larval thyroid grafts adjacent to the pituitary will induce a precocious metamorphosis in tadpoles,[71] a change that is known to depend on thyroid hormone [72]

It is interesting to note that successful endocrine homografts in mammals, although not common, seem to have taken without specific suppression of the

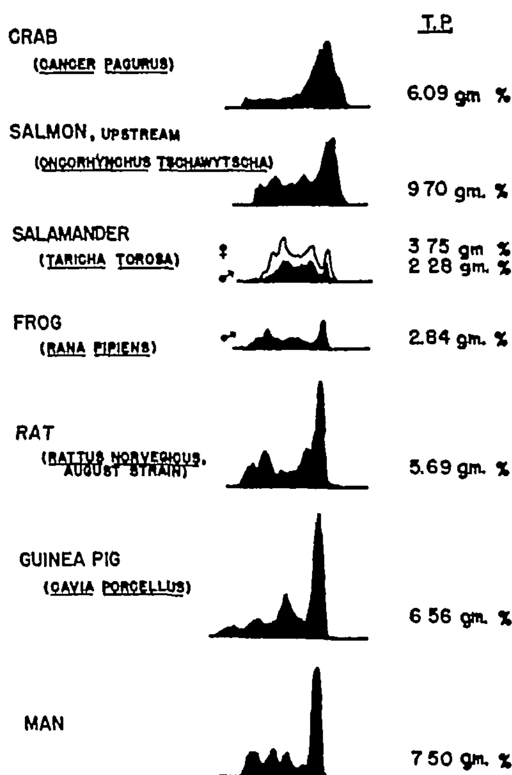



FIGURE 1 Comparative serum electrophoretic patterns of different phyla

TABLE 5
SUCCESSFUL HOMOTRANSPLANTATION

| | |
|---|---|
| *Invertebrates* | |
| Earthworm (Lumbricidae) | Head to body |
| Caterpillar (*Galleria*, *Cecropia*) | Head to body<br>Legs |
| *Vertebrates* | |
| Fish | |
| Amphibia | |
| Embryo | Limb bud, eye |
| Larva | Skin, thyroid |
| Birds | |
| Embryo | RE cells |
| Day-old chick | Skin |
| Mammals | |
| Embryo | RE cells |
| Day-old mouse | Cells, skin |
| Adults | ± Endocrines |

immune system of the recipient [73, 74] In mammals, perhaps, endocrine homografts more nearly represent the growth interrelations between graft and other tissues that prevail generally in invertebrates and that are recapitulated in homografts in larval amphibia and in chimeras created in mammalian embryos

*Discussion* This review of comparative immunology indicates that, in invertebrates that can be homografted, the plasma-cell system is absent, and the capacity to make gamma-type serum globulins is vestigial It is a provocative coincidence that vertebrates, which possess both plasma cells and gamma-like serum globulins, cannot readily be homografted[75, 76] except in their larval,[77, 78] embryonic,[79] or hypogammaglobulinemic states [2]

The role played by the lymphoid cell in homograft rejection is a matter for speculation Invertebrates that can be homografted do not possess non-phagocytic lymphoid cells like those seen in mammals, neither does the granuloma due to tubercle bacilli in insects develop giant cells, fibrosis, calcification, and symbiosis of bacilli within phagocytes,[80] as it does in vertebrates On the other hand, humans with hypogammaglobulinemia can be homografted,[2] even though they possess apparently normal lymphoid cells and a normal ability to acquire delayed-type allergy [81] It is interesting that experiments in which lymph node transfer has accelerated homograft rejection[82] are not successful when exudate cells are used Perhaps this is due to the presence of plasma cells in the former and not in the latter material These data suggest that delayed-type allergy, if it is a part of homograft rejection, by itself is not enough to cause the rejection of foreign tissue

The role of phagocytic cells in homograft rejection is equally difficult to ascertain Cellophane barriers about grafts, which pass serum proteins but not leukocytes, have been shown to protect homografts [83] On the other hand, humoral immunity alone, without local cellular inflammation,[84, 85] may be responsible for homograft failure

No doubt there are many reasons for homograft rejection The purpose of this report on comparative immunology and homotransplantation is to draw the attention of the student of human transplantation to lower animals as experimental tools for the analysis of the many factors that condition the success of homografting procedures

*References*


1 Bricker, N S, W R Guild, J B Reardan & J P Merrill 1956 Studies on the functional capacity of a denervated homotransplanted kidney in an identical twin with parallel observations in the donor J Clin Invest **35** 1364

2 Good, R A & R L Varco 1955 Successful homograft of skin in a child with agammaglobulinemia J Am Med Assoc **187** 713

3 Escamilla, R F, C H Kempe, J Crane, L Goldman & G S Gordan 1957 Preliminary experiences with transplants of cultured parathyroid tissue in hypoparathyroidism Ann Intern Med **46** 649

4 Ferrebee, J & J P Merrill 1957 Spare parts a review with a forward look Surgery **41** 503

5 Terasaki, P I, W P Longmire, Jr & J A Cannon 1957 The effect of size and donor age on survival of skin homografts in newly hatched chicks Transplantation Bull **4** 112

6 Woodruff, M F A 1957 Postpartum induction of tolerance to homologous skin in rats Ann N Y Acad Sci **64**(5) 792

7 Rabinovici, N 1947 The fate of skin homotransplants performed on previously x-rayed rats Plastic Reconstr Surg **2** 413

8 Odell, T T, Jr, F G Tausche, D L Lindsley & R D Owen 1957 The homotransplantation of functional erythropoietic elements in the rat following total body irradiation Ann N Y Acad Sci **64**(5) 811

9 BILLINGHAM, R E, L BRENT & P B MEDAWAR 1953 Actively acquired tolerance of foreign cells Nature **172** 603
10 WOODRUFF, M F A & L O SIMPSON 1955 Induction of tolerance to skin homografts in rats by injection of cells from the prospective donor shortly after birth Brit J Exptl Pathol **36** 494
11 BILLINGHAM, R E & L BRENT 1957 A simple method for inducing tolerance of skin homografts in mice. Transplantation Bull **4** 67
12 OWEN, R. D 1945 Immunogenetic consequences of vascular anastomosis between bovine twins Science **102** 400
13 DUNSFORD, I, C C BOWLEY, A. M HUTCHISON, J G THOMPSON, R SANGER & R R ROCA 1953 A human blood group chimera Brit Med J **2** 81
14 HASEK, M 1957 Embryonic parabiosis and related problems of immunological tolerance Transplantation Bull **4** 113
15 TRENTIN, J J 1957 The immunological basis for induced tolerance to skin homografts in irradiated mice receiving bone marrow transfusions Transplantation Bull **4** 74
16 FELTON, L D, G KAUFFMANN, B PRESCOTT & B OTTINGER 1955 Studies on the mechanism of the immunological paralysis induced in mice by pneumococcal polysaccharides J Immunol **74** 17
16a CHASE, M W 1946 Inhibition of experimental drug allergy by prior feeding of the sensitizing agent Proc Soc Exptl Biol Med **16** 257
17 HARDIN, C A. & A. A. WERDER 1955 Effect of skin extracts on the viability of homologous skin grafts in mice Ann N Y Acad Sci **59**(3) 381
18 SNELL, G D 1952 Enhancement and inhibition of the growth of tumor homotransplants by pretreatment of the hosts with various preparations of normal and tumor tissue J Natl Cancer Inst **13** 719
19 MITCHISON, N A & O L DUBE 1955 Studies on the immunological response to foreign tumor transplants in the mouse II The relation between hemagglutinating antibody and graft resistance in the normal mouse and mice pretreated with tissue preparations J Exptl Med **102** 179
20 METCHNIKOFF, E 1884 Über eine Sprosspilzkrankheit der Daphnien (On phagocytosis in *Daphnia*) Arch pathol Anat u Physiol Virchow's **96** 177
21 CHORINE, V 1931 Contribution à l'étude de l'immunité chez les insectes Bull biol France et Belg **65** 29
22 HUFF, C G 1940 Immunity in invertebrates Phys Rev **20** 68
23 NUNGESTER, W J 1951 Mechanisms of man's resistance to infectious diseases Bacteriol Rev **15** 105
24 CAMERON, G R 1934 Inflammation in the caterpillars of Lepidoptera J Pathol Bacteriol **38** 441
25 METALNIKOV, S 1927 L'infection microbienne et l'immunité chez la mite des abeilles Monogr inst Pasteur Paris
26 IWASAKI, Y 1926 L'encystement de corps étrangers aseptiques chez la chénille de Galléria Compt rend soc. biol **95** 98
27 CAMERON, G R 1932 Inflammation in earthworms J Pathol Bacteriol **35** 933
28 LEWIS, W H 1925 Transformation of mononuclear blood cells into macrophages, epithelioid cells and giant cells Harvey Lectures Ser **21** 77
29 SABIN, F R & C A DOAN 1927 Relation of monocytes and clasmatocytes to early infection in rabbits with bovine tubercle bacilli J Exptl Med **46** 627
30 SELYE, H 1953 Free discussion *In* Mechanism of Inflammation 255 G Jasmin & A Roberts, Eds Acta, Inc Montreal, Canada
31 FELL, H B & E M BRIEGER 1947 The effect of phagocytosis on the growth and survival of avian tubercle bacilli in embryonic chicken tissue cultivated *in vitro* J Hyg **45** 359
32 DOWNEY, H D 1938 Handbook of Hematology 725 Hoeber New York, N Y
33 GOOD, R. A. 1957 Morphological basis of the immune response and hypersensitivity *In* Host-Parasite Relationships in Living Cells 78 H M Felton, Ed Thomas Springfield, Ill
34 CHORINE, V 1929 Immunité antitoxique chez les chénilles de Galléria mellonella Ann inst Pasteur **43** 955
35 ZERNOFF, V 1928 Sur la nature de l'immunité passive chez les chénilles de Galléria mellonella Compt rend soc biol **99** 315
36 ZERNOFF, V 1928 Sur la spécificité de l'immunité passive chez Galléria mellonella Compt rend soc biol **98** 1500
37 PAILLOT, A 1920 L'immunité acquise chez les insectes Compt rend soc biol **83** 278

38 BISSET, K A. 1948 Natural antibodies in blood serum of fresh-water fish J Hyg **46** 267
39 SMITH, W W 1940 Production of antibacterial agglutinins by carp and trout at 10° C Proc Soc Exptl Biol Med **45** 726
40 ASHHURST, D E 1956 Red-blood-cell antigens in some lower vertebrates J Exptl Biol **33** 249
41 BOND, G C 1939 Serological studies of the reptilia I Hemagglutinins and hemagglutinogens of snake blood J Immunol **36** 1
42 BOND, G C 1940 Serological studies of the reptilia IV Hemagglutinins and hemagglutinogens of alligator blood J Immunol **39** 136
43 BOND, G C 1940 Serological studies of the reptiles III Hemagglutinins and hemagglutinogens of turtle blood J Immunol **39** 125
44 GRASSET, E & A ZOUTENDIJK 1931 Immunological studies in reptiles and their relation to aspects of immunity in higher animals S African Inst Med Research **29.** 377
45 DREYER, N B & J W KING 1948 Anaphylaxis in the fish J Immunol **60** 277
46 DOWNS, C M 1928 Anaphylaxis active anaphylaxis in turtles J Immunol **15** 77
47 KING, J W & N B DREYER 1949 Hypersensitivity in cold-blooded animals (*Trit virid* and *Necturus*) II Salamander Science **109** 568
48 CUSHING, J R 1942 The effect of temperature on antibody formation in fish J Immunol **45** 123
49 ALLEN, F W & E C MCDANIEL 1937 A study of the relation of temperature to antibody formation in cold-blooded animals J Immunol **32** 142
50 KAY, C F 1940 Mechanism by which experimental nephritis is produced in rabbits injected with nephrotoxic duck serum J Exptl Med **72** 559
51 EWING, J 1903 Differentiation of monkey and human blood by the serum test Proc N Y Pathol Soc **3** 14
52 RICH, A R 1944 Pathogenesis of Tuberculosis 33 Thomas Springfield, Ill
53 EHRICH, W E 1953 Adaptation phase in inflammation Rev can biol **12** 127
54 FAVOUR, C B 1954 Effect of adrenalectomy on tuberculin reaction in mice Proc Soc Exptl Biol Med **85** 237
55 FAVOUR, C B 1957 Cell injury in allergic inflammation Intern Arch Allergy Appl Immunol **10** 193
56 SWEETMAN, H L 1936 Biological Control of Insects Comstock New York, N Y
57 HARRIS, S, T N HARRIS, C A OGBURN & M B FARBER 1956 Studies on transfer of lymph node cells VII. Transfer of cells incubated *in vitro* with filtrates of trypsin-treated suspensions of *Shigella paradysenteriae* J Exptl Med **104** 645
58 ORTEGA, L B & R C MELLORS 1957 Cellular sites of formation of globulin J Exptl Med **106** 627
59 FAGRAEUS, A 1948 Antibody production in relation to the development of plasma cells *in vivo* and *in vitro* experiments (Translated by Ulla Schott) Acta Med Scand Suppl 204 **130** 122
60 COONS, A H, E H LEDUC & J M CONNOLLY 1955 Studies on antibody production I A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit J Exptl Med **102** 49
61 DURRUM, E L 1950 A microelectrophoretic and microionophoretic technique J Am Chem Soc **72** 2943
62 DESSAUER, H C & W FOX 1956 Characteristic electrophoretic patterns of plasma proteins of orders of amphibia and reptilia Science **124** 225
63 BORN, G 1897 Über Verwachsungsversuche mit Amphibien-larven Wilhelm Roux' Arch Entwicklungsmech Organ **4** 349
64 PIEPKO, H 1939 Raupenhäutungen bereits verpupter Hauptstücke bei der Wachsmotte *Galleria mellonella* Naturwiss **27** 301
65 FURUKOWA, H 1935 Can the skin of imago be made to molt? Proc Imp Acad Japan **11** 158
66 BODENSTEIN, D 1933 Beintransplantationen an Lepidopteren-raupen I Wilhelm Roux' Arch Entwicklungsmech Organ **128** 564
67 WILLIAMS, C 1952 Physiology of insect diapause Biol Bull **103** 120
68 TURNER, C 1948 General Endocrinology Saunders Philadelphia, Pa
69 WEISS, P 1936 Selectivity controlling the central-peripheral relations in the nervous system Biol Revs Cambridge Phil Soc **11** 494
70 WEISS, P 1952 Central versus peripheral factors in the development of coordination Assoc Nervous Mental Disease **30** 3

71 SWINGLE, W 1923 Thyroid transplantation and anural metamorphosis J Exptl Zool **37** 219
72 SLOWEKOWSKA, S 1923 Recherches expérimentales sur le rôle de la glande thyroïde dans la métamorphose des Batraciens Compt rend soc biol **89** 1396
73 STEVENS, L C 1957 A modification of Robertson's technique of homoiotopic ovarian transplantation in mice Transplantation Bull **4** 106
74 GREENE, H S N 1955 Compatability and noncompatability Ann N Y Acad Sci **59**(3) 311
75 VOGEL, H H., JR 1940 Autoplastic and homoplastic transplantation of skin in adult *Rana pipiens* Schreber J Exptl Zool **85** 437
76 COLE, W H 1922 Transplantation of skin in frog tadpoles, with special reference to the adjustment of grafts over eyes, and to local specificity of integument J Exptl Zool **35** 353
77 DUNIHUE, F W 1934 Histolysis and regeneration of anuran tail skin Biol. Bull **67** 381
78 LINDEMAN, V F 1929 Transplantation and metamorphosis of frogskin Physiol. Zool **2** 255
79 TWITTY, V 1955 Eye. *In* Analysis of Development 402 B H Willier, P A Weiss & V Hamburger, Eds Saunders Philadelphia, Pa
80 HOLLANDE, A. C & M GELY 1929 La formation des nodules leucocytaires tuberculeux chez l'insecte Compt rend soc biol. **102** 384
81 PORTER, H M 1956 The demonstration of delayed-type reactivity in congenital agammaglobulinemia Ann N Y Acad Sci **64**(5) 8
82 MITCHISON, N A. 1955 Studies on the immunological response to foreign tumor transplants in the mouse I The role of lymph node cells in conferring immunity by adoptive transfer J Exptl Med **102** 157
83 ALGIRE, G H, J M WEAVER & R. T PREHN 1956 Studies on tissue homotransplantation in mice using diffusion chamber methods Ann N Y Acad Sci **64**(5) 15
84 WOODRUFF, M F A. 1957 Cellular and humoral factors in the immunity to skin homografts experiments with a porous membrane. Ann N Y Acad Sci **64**(5) 1014
85 AMOS, D B & E D DAY 1957 Passive immunity against four mouse leukoses by means of isoimmune sera Ann N Y Acad Sci **64**(5) 851
86 LANNI, F, M L DILLON & J W BEARD 1950 Determination of small quantities of nitrogen in serological precipitates and their biological materials Proc. Soc Exptl Biol Med **74** 4
87 OBERMAN, J W, O KRIKOR, B GREGORY, F G BURKE, S ROSS & C RICE 1956 Normal values from birth to adolescence (electrophoresis) New Engl J Med **255** 743

# GENETICS OF FIN TRANSPLANTATION IN XIPHOPHORIN FISHES*

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## Introduction

Successful transplantations of fins between members of one highly inbred strain of platyfish, *Xiphophorus maculatus*, were shown to be feasible by Kallman and Gordon (1957). Since then, a second highly inbred strain of platyfish was developed in our laboratory that has provided the necessary material for an analysis of the genetic constitution of host and donor in determining whether a graft will be accepted or rejected.

The present concept that the fate of a transplant depends upon histocompatibility genes is derived largely from experiments with inbred strains of mice and rats. It is desirable to determine if this genetic concept is applicable to other classes of vertebrates, especially the fishes. This paper is a report of some results that we have obtained thus far from our experiments with the transplantation of fins among members of two highly inbred strains of platyfish and their $F_1$ hybrids.

## Material and Methods

Our two strains of the platyfish *Xiphophorus maculatus* originated from a single random sample of the platyfish population collected in 1939 from the Río Jamapa in Mexico. Strain 30 has been inbred by brother-sister matings since 1939 for 25 generations. The other, strain 163, derived from the same sample, has been inbred for only 12 generations. These two strains are quite distinct now and differ in their pigment patterns. The $F_1$ generation obtained from crossing members of 30 ♀ and 163 ♂ bears the pedigree number 784, their $F_2$ generation is 793. All fish were maintained in laboratory aquaria in a manner described by Gordon (1950). Water temperature varied between 21° and 27° C.

We transplanted anal, caudal, and dorsal fins according to a method described by Kallman and Gordon (1957). First, the fin, including part of its suspensorium, is dissected from the donor and placed into a dish containing a modified Ringer's solution, and then the host fish is placed on a piece of wet cotton in a Syracuse watch glass and held in position by another piece of wet cotton placed over the head region. The scales of the host fish near the selected site of implantation are removed and a slit, or pocket, is made in its musculature by means of a fine glass needle. This incision succeeds most easily just above the origin of the anal fin where the musculature is well developed (FIGURES 1 and 2), however, fins have been transplanted on both sides of the dorsal fin, anterior to the dorsal fin, and in the caudal peduncle near

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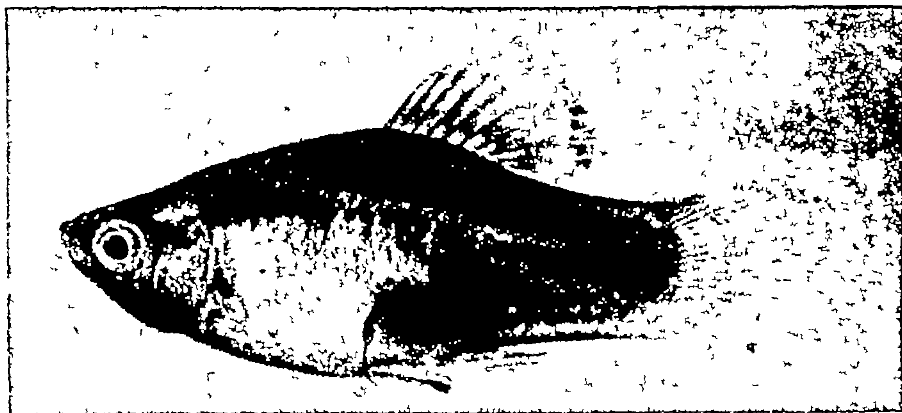


FIGURE 1 An adult male platyfish of strain 163 into which a caudal fin with part of the caudal peduncle had been transplanted Photograph taken 4 months after the operation × 2 7

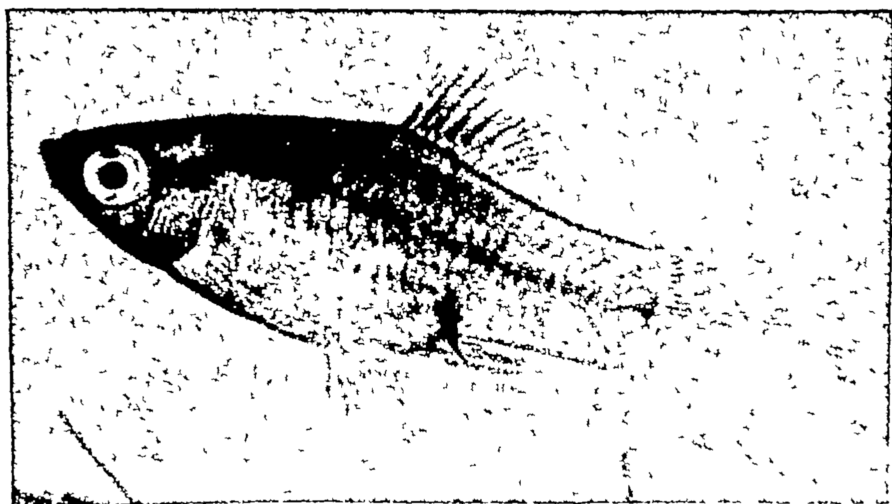


FIGURE 2 An adult male platyfish of strain 163 into which a dorsal fin of a sibling had been transplanted Photograph taken 9 months after the operation ×2 7

the tail fin Subsequently, the fin to be transplanted is transferred from the dish containing Ringer's solution to the site of implantation, then the proximal portion of the donor's fin is squeezed into the slit With some practice the time required for one complete transplantation operation may be reduced to less than three minutes Provided the host fish is in vigorous good health, the frequency of postoperative mortality is below 1 per cent No anesthetic or antiseptic is required After the operation, to inhibit bacterial infection, the

fish were maintained for 5 days in conditioned aquarium water to which 6 gm of coarse household rock salt per 1000 cc was added

In our experiments a fin graft was judged successful when the transplanted fin grew and increased in size while the host lived out its normal life span of 2 years or more Some of these fish were used for further genetic experiments

## Results

### *Intrastrain Grafts (Isografts)*

Thirty-three dorsal, anal, or caudal fins were transplanted between members of inbred strain 30, and 33 others between members of strain 163 In each transplantation experiment the hosts and donors were siblings, their age at the time of the operation varied from 5 to 81 days All 66 were completely successful (TABLE 1)

It was observed that the graft undergoes certain characteristic changes within the first 2 weeks after a fin is transplanted Two to 8 days after the operation the graft becomes vascularized, and this is indicated by the greatly distended longitudinal artery and the blood vessels of the fin rays This vascular engorgement persists for 2 to 3 days Next, the distal portion of the transplanted fin sloughs off by a progressive degeneration of its distal margin The thin, soft membranes between the fin rays lose their natural resilience, the pigment cells disintegrate, and then the tissues slough The fin rays may persist for 1 or 2 days, but ultimately they too disappear The fin tissues at the base, however, maintain their normal appearance A new fin regenerates from the basal stump within 12 to 25 days after the operation The sloughing process may stop at any level of the fin, but it usually proceeds down to the base of the transplant, leaving only a narrow fringe of normal tissue projecting from the pocket, in some cases no sloughing takes place There is no obvious relationship between the onset of vascularization and the sloughing process Of-

TABLE 1

FIN TRANSPLANTS IN THE PLATYFISH *XIPHOPHORUS MACULATUS*

| Donor | Host | Age of host, days | Number of transplants | Type | Success | Failure |
|---|---|---|---|---|---|---|
| 30* | 30 | 81 | 16 | Intrastrain | 16 | 0 |
| 30 | 30 | 5 | 17 | Intrastrain | 17 | 0 |
| 163† | 163 | 35 | 23 | Intrastrain | 23 | 0 |
| 163 | 163 | 10 | 10 | Intrastrain | 10 | 0 |
| 30 | $F_1$‡ | 10 | 31 | $P_1$ to $F_1$ | 31 | 0 |
| 163 | $F_1$ | 10 | 30 | $P_1$ to $F_1$ | 30 | 0 |
| $F_1$ | $F_1$ | 10 | 22 | $F_1$ to $F_1$ | 22 | 0 |
| $F_1$ | $F_1$ | 62 | 10 | $F_1$ to $F_1$ | 10 | 0 |
| 30 | 163 | 10 | 60 | Interstrain | 0 | 60 |
| 163 | 30 | 10 | 40 | Interstrain | 0 | 40 |
| $F_1$ | 30 | 10 | 28 | $F_1$ to $P_1$ | 0 | 28 |
| $F_1$ | 163 | 10 | 32 | $F_1$ to $P_1$ | 0 | 32 |

* Strain 30 was inbred 24 generations by brother-sister matings
† Strain 163 was inbred 12 generations by brother-sister matings
‡ $F_1$ = pedigree number 784 30 × 163

ten, when vascularization in the fin begins on the second day after the transplantation and is completed on the fourth, a few days may intervene before the sloughing process sets in and destroys the distal portion of an apparently normal fin. If vascularization is late, the sloughing process may proceed simultaneously and be accompanied by hemorrhages and capillary leakages at the fin's distal margin.

### *Transplants from Parental Strains 30 and 163 to Their $F_1$ Hybrids and Between $F_1$ Hybrids 784*

Sixty-one dorsal or anal fins were transplanted from donors of strain 30 or 163 into their $F_1$ offspring (784), and 32 fins were removed and transplanted among the $F_1$ members. All 93 transplants were successful (TABLE 1).

In all transplants made, the hosts and donors involved were first cousins and of approximately the same age, the fishes were from 10 to 62 days old. The sequence and incidence of sloughing of these transplanted fins were the same as indicated for the intrastrain grafts.

### *Interstrain Grafts (Homografts)*

*Primary grafts.* Sixty fin transplants were attempted from strain 30 donors into strain 163 hosts and, in the reciprocal experiment, 40 transplants were made from 163 donors into strain 30 hosts. All 100 grafts were rejected

TABLE 2

STAGES IN THE BREAKDOWN OF 38 HOMOGRAFTS FROM STRAIN 30 DONORS TO STRAIN 163 HOST PLATYFISH

| Condition of graft | Days after transplantation | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| No change | 38 | 38 | 34 | 22 | 14 | 9 | 5 | 2 | 1 | 1 | 1 | 1 | 0 | 0 | 0 |
| Sloughing | 0 | 0 | 4 | 13 | 14 | 13 | 8 | 6 | 2 | 1 | 0 | 0 | 0 | 0 | 0 |
| Base left | 0 | 0 | 0 | 2 | 4 | 7 | 7 | 7 | 5 | 4 | 2 | 2 | 0 | 0 | 0 |
| Regenerating | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Degenerating | 0 | 0 | 0 | 1 | 5 | 6 | 7 | 4 | 6 | 4 | 2 | 1 | 1 | 1 | 0 |
| Disintegrated | 0 | 0 | 0 | 0 | 1 | 3 | 11 | 19 | 24 | 28 | 33 | 34 | 37 | 37 | 38 |

TABLE 3

STAGES IN THE BREAKDOWN OF THIRTY HOMOGRAFTS FROM STRAIN 163 DONORS TO STRAIN 30 HOST PLATYFISH

| Condition of graft | Days after transplantation | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| No change | 30 | 30 | 26 | 19 | 12 | 6 | 5 | 4 | 2 | 1 | 0 | 0 | 0 | 0 |
| Sloughing | 0 | 0 | 4 | 11 | 13 | 12 | 8 | 2 | 1 | 0 | 0 | 0 | 0 | 0 |
| Base left | 0 | 0 | 0 | 0 | 2 | 4 | 5 | 5 | 4 | 1 | 1 | 1 | 0 | 0 |
| Regenerating | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Degenerating | 0 | 0 | 0 | 0 | 3 | 7 | 8 | 11 | 10 | 10 | 3 | 2 | 1 | 0 |
| Disintegrated | 0 | 0 | 0 | 0 | 0 | 1 | 4 | 8 | 13 | 18 | 26 | 27 | 29 | 30 |

between the fifth and fifteenth days, but mostly between the seventh and eleventh days after the operation (TABLES 2 and 3)

The first sign of an immunological reaction was an extensive edema around the pocket holding the proximal portion of the graft that occurred between the fifth and tenth days, it was sometimes so extreme that the graft was literally pushed out of its pocket Next, the soft tissue between the fin rays and their pigment cells disintegrated at all levels, and this was followed by a breakdown of the fin rays close to their bases Once the homograft reaction set in, the fin graft was destroyed within 2 or 3 days

*Secondary grafts* Twenty-eight days later, a second fin from the same donor strain was transplanted into each host Thirty fins from strain 163 donors were transplanted as secondary grafts into strain 30 hosts In reciprocal experiments thirty strain 163 hosts received secondary fins from strain 30 donors All 60 secondary grafts were rejected within 2 to 7 days, the majority between the third and fifth day (TABLES 4 and 5) In many transplants the homograft reaction set in before vascularization and, once begun, the transplant was destroyed within 1 or 2 days (the fate of these 60 secondary interstrain grafts is not included in TABLE 1, because it lists primary grafts only)

### *Transplants from $F_1$ Hybrids to Each of the $P_1$ Strains*

Twenty-eight fins were transplanted from the $F_1$ members (pedigree 784) into strain 30 $P_1$ hosts, and 32 fins into strain 163 $P_1$ hosts Although all grafts eventually degenerated, a great difference between the survival times of the individual grafts was observed In the strain 30 hosts one transplant

TABLE 4
STAGES IN THE BREAKDOWN OF THIRTY SECONDARY HOMOGRAFTS FROM STRAIN 30 DONORS TO STRAIN 163 HOST PLATYFISH

| Condition of graft | Days after transplantation | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| No change | 30 | 23 | 13 | 1 | 0 | 0 | 0 | 0 |
| Degenerating | 0 | 6 | 7 | 10 | 2 | 1 | 0 | 0 |
| Disintegrated | 0 | 1 | 10 | 19 | 28 | 29 | 30 | 30 |

TABLE 5
STAGES IN THE BREAKDOWN OF THIRTY SECONDARY HOMOGRAFTS FROM STRAIN 163 DONORS TO STRAIN 30 HOST PLATYFISH

| Condition of graft | Days after transplantation | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| No change | 30 | 24 | 7 | 0 | 0 | 0 | 0 |
| Degenerating | 0 | 6 | 15 | 7 | 1 | 0 | 0 |
| Disintegrated | 0 | 0 | 8 | 23 | 29 | 30 | 30 |

ten, when vascularization in the fin begins on the second day after the transplantation and is completed on the fourth, a few days may intervene before the sloughing process sets in and destroys the distal portion of an apparently normal fin If vascularization is late, the sloughing process may proceed simultaneously and be accompanied by hemorrhages and capillary leakages at the fin's distal margin

### *Transplants from Parental Strains 30 and 163 to Their $F_1$ Hybrids and Between $F_1$ Hybrids 784*

Sixty-one dorsal or anal fins were transplanted from donors of strain 30 or 163 into their $F_1$ offspring (784), and 32 fins were removed and transplanted among the $F_1$ members All 93 transplants were successful (TABLE 1)

In all transplants made, the hosts and donors involved were first cousins and of approximately the same age, the fishes were from 10 to 62 days old The sequence and incidence of sloughing of these transplanted fins were the same as indicated for the intrastrain grafts

### *Interstrain Grafts (Homografts)*

*Primary grafts* Sixty fin transplants were attempted from strain 30 donors into strain 163 hosts and, in the reciprocal experiment, 40 transplants were made from 163 donors into strain 30 hosts All 100 grafts were rejected

TABLE 2
STAGES IN THE BREAKDOWN OF 38 HOMOGRAFTS FROM STRAIN 30 DONORS TO STRAIN 163 HOST PLATYFISH

| Condition of graft | Days after transplantation | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| No change | 38 | 38 | 34 | 22 | 14 | 9 | 5 | 2 | 1 | 1 | 1 | 1 | 0 | 0 | 0 |
| Sloughing | 0 | 0 | 4 | 13 | 14 | 13 | 8 | 6 | 2 | 1 | 0 | 0 | 0 | 0 | 0 |
| Base left | 0 | 0 | 0 | 2 | 4 | 7 | 7 | 7 | 5 | 4 | 2 | 2 | 0 | 0 | 0 |
| Regenerating | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Degenerating | 0 | 0 | 0 | 1 | 5 | 6 | 7 | 4 | 6 | 4 | 2 | 1 | 1 | 1 | 0 |
| Disintegrated | 0 | 0 | 0 | 0 | 1 | 3 | 11 | 19 | 24 | 28 | 33 | 34 | 37 | 37 | 38 |

TABLE 3
STAGES IN THE BREAKDOWN OF THIRTY HOMOGRAFTS FROM STRAIN 163 DONORS TO STRAIN 30 HOST PLATYFISH

| Condition of graft | Days after transplantation | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| No change | 30 | 30 | 26 | 19 | 12 | 6 | 5 | 4 | 2 | 1 | 0 | 0 | 0 | 0 |
| Sloughing | 0 | 0 | 4 | 11 | 13 | 12 | 8 | 2 | 1 | 0 | 0 | 0 | 0 | 0 |
| Base left | 0 | 0 | 0 | 0 | 2 | 4 | 5 | 5 | 4 | 1 | 1 | 1 | 0 | 0 |
| Regenerating | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Degenerating | 0 | 0 | 0 | 0 | 3 | 7 | 8 | 11 | 10 | 10 | 3 | 2 | 1 | 0 |
| Disintegrated | 0 | 0 | 0 | 0 | 0 | 1 | 4 | 8 | 13 | 18 | 26 | 27 | 29 | 30 |

between the fifth and fifteenth days, but mostly between the seventh and eleventh days after the operation (TABLES 2 and 3)

The first sign of an immunological reaction was an extensive edema around the pocket holding the proximal portion of the graft that occurred between the fifth and tenth days, it was sometimes so extreme that the graft was literally pushed out of its pocket Next, the soft tissue between the fin rays and their pigment cells disintegrated at all levels, and this was followed by a breakdown of the fin rays close to their bases Once the homograft reaction set in, the fin graft was destroyed within 2 or 3 days

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### *Transplants from $F_1$ Hybrids to Each of the $P_1$ Strains*

Twenty-eight fins were transplanted from the $F_1$ members (pedigree 784) into strain 30 $P_1$ hosts, and 32 fins into strain 163 $P_1$ hosts Although all grafts eventually degenerated, a great difference between the survival times of the individual grafts was observed In the strain 30 hosts one transplant

TABLE 4
STAGES IN THE BREAKDOWN OF THIRTY SECONDARY HOMOGRAFTS FROM STRAIN 30 DONORS TO STRAIN 163 HOST PLATYFISH

| Condition of graft | Days after transplantation | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| No change | 30 | 23 | 13 | 1 | 0 | 0 | 0 | 0 |
| Degenerating | 0 | 6 | 7 | 10 | 2 | 1 | 0 | 0 |
| Disintegrated | 0 | 1 | 10 | 19 | 28 | 29 | 30 | 30 |

TABLE 5
STAGES IN THE BREAKDOWN OF THIRTY SECONDARY HOMOGRAFTS FROM STRAIN 163 DONORS TO STRAIN 30 HOST PLATYFISH

| Condition of graft | Days after transplantation | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| No change | 30 | 24 | 7 | 0 | 0 | 0 | 0 |
| Degenerating | 0 | 6 | 15 | 7 | 1 | 0 | 0 |
| Disintegrated | 0 | 0 | 8 | 23 | 29 | 30 | 30 |

TABLE 6

STAGES IN THE BREAKDOWN OF TWENTY-EIGHT HOMOGRAFTS FROM $F_1$ DONORS (30 × 163) TO STRAIN 30 HOST PLATYFISH

| Condition of graft | Days after transplantation | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22* |
| No change | 28 | 28 | 27 | 21 | 11 | 8 | 7 | 4 | 4 | 2 | 2 | 2 | 2 | 2 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Sloughing | 0 | 0 | 1 | 7 | 14 | 14 | 13 | 11 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Base left | 0 | 0 | 0 | 0 | 3 | 3 | 3 | 7 | 16 | 16 | 14 | 14 | 11 | 7 | 7 | 6 | 6 | 4 | 3 | 3 | 3 | 3 |
| Regenerating | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 3 | 3 | 3 | 3 | 3 |
| Degenerating | 0 | 0 | 0 | 0 | 0 | 3 | 4 | 5 | 4 | 5 | 7 | 6 | 7 | 8 | 8 | 6 | 5 | 3 | 1 | 1 | 1 | 0 |
| Disintegrated | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 3 | 3 | 4 | 5 | 7 | 10 | 11 | 13 | 14 | 17 | 20 | 20 | 20 | 21 |

* For subsequent fate of homografts see TABLE 10

TABLE 7

STAGES IN THE BREAKDOWN OF THIRTY-TWO FIN HOMOGRAFTS FROM $F_1$ DONORS (30 × 163) TO STRAIN 163 HOST PLATYFISH

| Condition of graft | Days after transplantation | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 22 | 24 | 28 |
| No change | 32 | 32 | 32 | 29 | 27 | 24 | 15 | 11 | 7 | 4 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Sloughing | 0 | 0 | 0 | 3 | 5 | 7 | 11 | 8 | 9 | 8 | 4 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Base left | 0 | 0 | 0 | 0 | 0 | 1 | 4 | 6 | 10 | 13 | 19 | 19 | 17 | 15 | 12 | 8 | 7 | 4 | 4 | 2 | 2 | 1 | 1* |
| Regenerating | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 3 | 7 | 7 | 6 | 5 | 5 | 4 | 4 | 4* |
| Degenerating | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 6 | 6 | 5 | 6 | 7 | 9 | 10 | 10 | 9 | 9 | 10 | 7 | 6 | 4 | 4 | 0 |
| Disintegrated | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 3 | 6 | 6 | 7 | 8 | 10 | 12 | 16 | 19 | 20 | 23 | 27 |

* Survived until the 37th, 42nd, 49th, 55th, and 67th day

completely disintegrated by the seventh day, while another survived until the fifty-seventh day (TABLE 6) In the strain 163 hosts the survival time of the transplants varied from 10 to 67 days (TABLE 7)

In 10 grafts that survived for 1 or 2 months, regeneration occurred after the sloughing process, but they too eventually disappeared Their incompatibility was first manifested only by the general retardation of the growth of the fin Much later the pigment cells in certain areas of the graft began to degenerate over a 2-week period, and then the remaining colorless fin disintegrated completely within 1 or 2 days

*Secondary grafts* Twenty strain 163 fish and 21 strain 30 fish in which the graft from the $F_1$ donor had completely degenerated on the 28th day received a secondary transplant from strains 30 and 163 donors, respectively (TABLES 8 and 9) Again the majority of the grafts broke down between the third and fifth days, and within a week all 41 grafts were completely rejected

Finally, the 6 strain 30 fish in which the primary graft from the $F_1$ donors had not yet broken down by the twenty-eighth day received a secondary graft from strain 163 donors (TABLE 10) In these experiments host fish 30 *A* did not receive a secondary graft and served as control, and it was determined that its primary $F_1$ graft survived until the forty-sixth day In

TABLE 8

STAGES IN THE BREAKDOWN OF TWENTY SECONDARY FIN HOMOGRAFTS FIRST DONOR $F_1$, SECOND DONOR STRAIN 30, HOST STRAIN 163, PLATYFISH

| Condition of graft | Days after transplantation | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| No change | 20 | 17 | 4 | 1 | 0 | 0 | 0 |
| Degenerating | 0 | 3 | 11 | 6 | 2 | 1 | 0 |
| Disintegrated | 0 | 0 | 5 | 13 | 18 | 19 | 20 |

TABLE 9

STAGES IN THE BREAKDOWN OF TWENTY-ONE SECONDARY FIN HOMOGRAFTS FIRST DONOR $F_1$, SECOND DONOR STRAIN 163, HOST STRAIN 30 PLATYFISH

| Condition of graft | Days after transplantation | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| No change | 21 | 19 | 12 | 2 | 0 | 0 | 0 |
| Degenerating | 0 | 2 | 6 | 11 | 5 | 1 | 0 |
| Disintegrated | 0 | 0 | 3 | 8 | 16 | 20 | 21 |

TABLE 10

EFFECT OF SECONDARY GRAFTS (DONOR STRAIN 163) ON THE RATE OF DISINTEGRATION OF PRIMARY GRAFTS (DONOR $F_1$), PLATYFISH

| Fish | Condition of primary graft on 28th day after transplantation | Fate of secondary graft, implanted 28 days after first graft | Fate of primary graft |
|---|---|---|---|
| A | Graft had sloughed off, then regeneration, condition good | None | Disintegrated on 46th day |
| B | No sloughing, fin in perfect condition | Degeneration beginning on third day and completed on fifth | Degeneration beginning on 42nd day and completed on 50th |
| C | After sloughing, regeneration from base, growth ceased around 25th day | Degeneration beginning on third day and completed on fifth | Gradual disintegration from 45th day on, survived until 57th day |
| D | After sloughing, regeneration from base, growth ceased around 25th day | Still in good condition on fifth day, but completely disintegrated on seventh day | Survived until 48th day |
| E | Only base left, no regeneration, some degeneration of pigment cells | Degenerated on third day | Degenerated on third day |
| F, G | Only base left, no regeneration, some degeneration of pigment cells | Degenerated on fifth day | Degenerated on 33rd day (same day as second-set grafts) |

30 *B*, while the primary graft was apparently still in perfect condition, its secondary graft degenerated within 5 days, the primary one survived until the fiftieth day In 30 *C* and *D* the secondary grafts broke down within a week, while the primary grafts survived until the forty-eighth and fifty-seventh day, respectively In 30 *E*, *F*, and *G* the primary grafts had sloughed off, regeneration had not occurred, and only their basal stumps persisted on the twenty-eighth day Their secondary grafts were rejected within 5 days The primary grafts of 30 *F* and *G* disintegrated on the same day as their secondary grafts, the primary graft of 30 *E* survived until the thirty-eighth day

*Grafts from the Parental ($P_1$) Inbred Strains into $F_2$ Hybrids*

A fin from a strain 30 donor was grafted on the right side of an $F_2$ fish (pedigree 793) and a fin from a strain 163 donor was grafted on its left side We have made more than 1000 of these transplants recently and it is not yet possible to determine in all cases whether a graft is successful or not A final analysis will be made in a later paper In our preliminary results it appears that only approximately one third of all transplants have taken, one third of the transplanted fins were rejected within 3 weeks after the operation, while another third gradually degenerated during the next 3 months

## Discussion

In view of the uniform acceptance of all intrastrain grafts from and to highly inbred lines of fishes, of all grafts from each $P_1$ to their $F_1$ hybrids, and of all grafts among members of the $F_1$ generation, while all the interstrain grafts and grafts from the $F_1$ hybrids to each $P_1$ were uniformly rejected, the results clearly indicate that the genetic constitution of the platyfish is of prime importance in determining the success or failure of the graft, just as it is in mice and other mammalian species (Snell, 1953) This can be seen in FIGURE 3 Transplants among members of highly inbred strains take because hosts and donors are identical genetically In platyfish, transplanted fins from each parent take in their $F_1$ hybrids because the $F_1$ fish possess one set of chromosomes in common with the $P_1$ fish of inbred strain 30 and another set of chromosomes in common with the $P_1$ fish of inbred strain 163 The fin from a parent strain does not introduce into the host any "foreign" genes via its chromosomes Transplants among the $F_1$ hybrids of two inbred strains also succeed, for the $F_1$ fish are all identical genetically

In interstrain grafts, however, both host and donor have quite different genetic constitutions Thus, a fin from a strain 30 fish transplanted into a strain 163 host introduces some histocompatibility genes that are absent in the recipient In the host these incompatible genes provoke a homograft reaction against the donor's graft Similarly, all fin and scale tissue transplants (with the exception of autotransplants) of Held (1951), Goodrich and Nichols (1933), Hildemann (1957), and others were rejected because the hosts and donors used were not of the same highly inbred strain

A somewhat similar situation occurs when fins from the $F_1$ platyfish hybrids are transplanted into members of each parental strain In this case only a

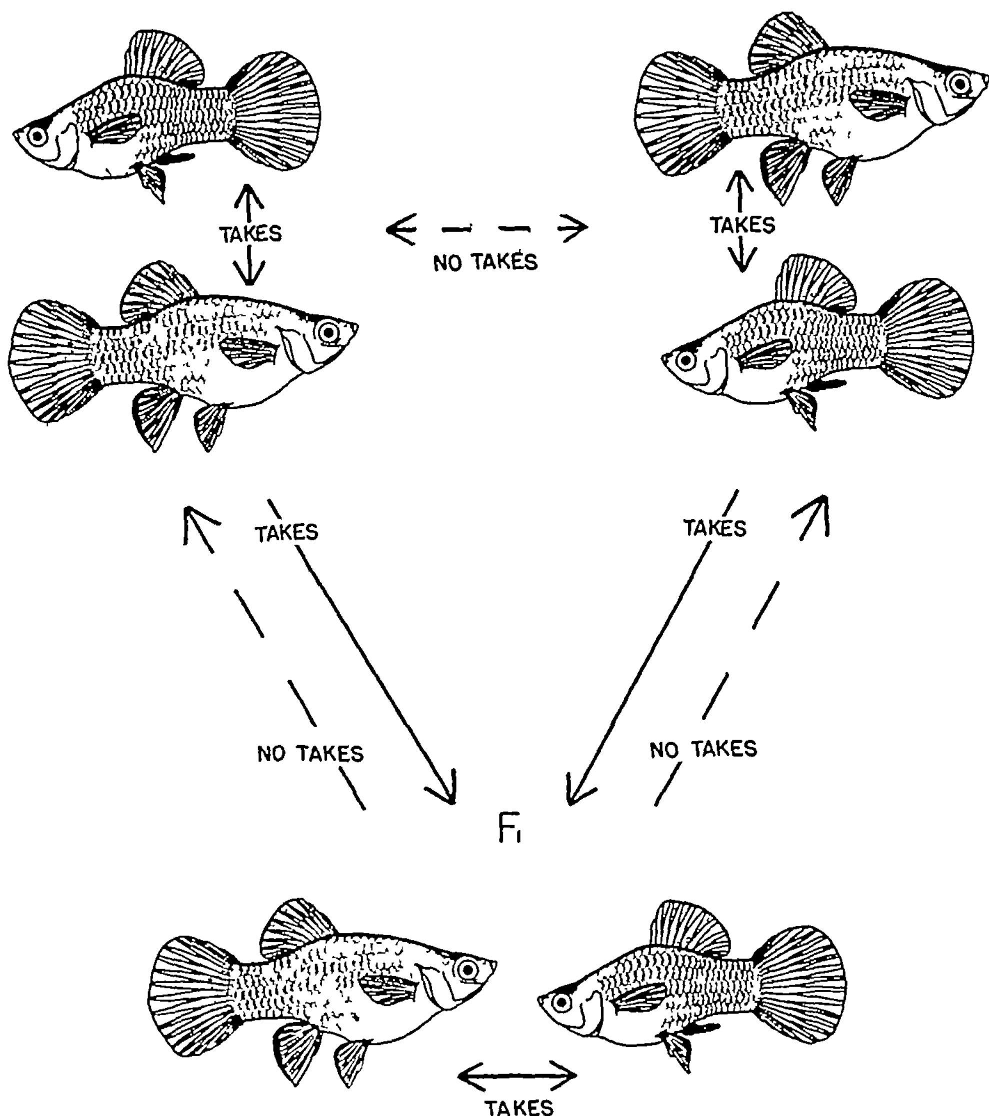



FIGURE 3 Diagram illustrating the genetics of fin transplantation The arrows point from the donors toward the hosts The solid arrows indicate that a transplant has been successful (a) from $P_1$ 30 to $P_1$ 30, (b) $P_1$ 163 to $P_1$ 163, (c) $F_1$ to $F_1$, (d) $P_1$ 30 to $F_1$, (e) $P_1$ 163 to $F_1$

The broken arrows indicate that a transplant has been rejected (a) from $P_1$ 30 to $P_1$ 163, (b) $P_1$ 163 to $P_1$ 30, (c) $F_1$ to $P_1$ 30, (d) $F_1$ to $P_1$ 163

single set of "foreign" chromosomes and their genes is introduced into the hosts, and this smaller genetic disparity is reflected in the prolonged survivals of the $F_1$ fin grafts in the parental strains 30 and 163 (FIGURE 4)

In the final analysis, the $F_1$ grafts were actually as effective in inducing an immunity reaction in the 30 and 163 strains as the interstrain grafts. This

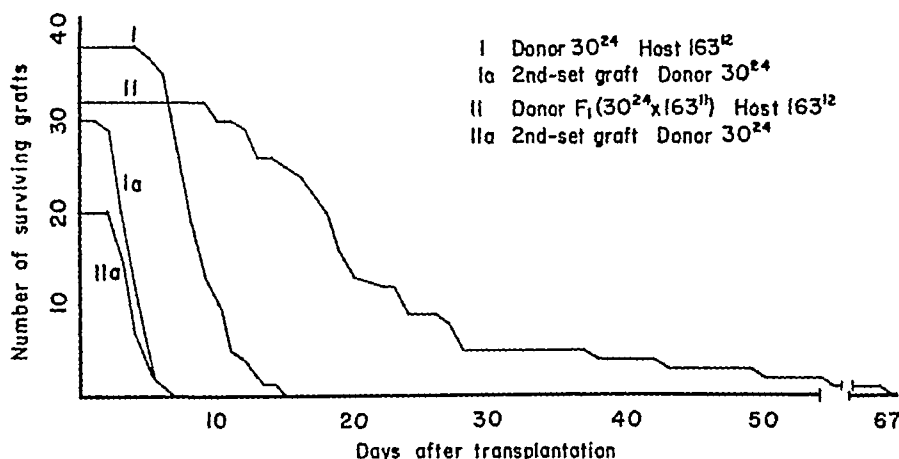



FIGURE 4 Comparison of survival times of first-set (primary) $30^{24}$ and $F_1$ grafts in $163^{12}$ hosts and second-set (secondary) $30^{24}$ grafts in same hosts The superscripts refer to the number of inbred generations

is clearly brought out by comparing the survival times of all secondary interstrain grafts Regardless of whether the primary graft had been an interstrain or an $F_1$ graft, the survival times of their secondary grafts were identical

The prolonged survival times of some of the $F_1$ grafts may be attributed to the failure of the grafts to elicit a strong homograft reaction in the host or the presence of one set of host-specific histocompatibility genes in each cell of the transplanted fins may inhibit, temporarily at least, the host's antagonistic reactions We have attempted to clarify this situation by transplanting a secondary strain 163 fin into strain 30 hosts in which the primary $F_1$ grafts were still present on the twenty-eighth day In all cases, regardless of whether the first graft was intact or degenerating, the secondary transplant disintegrated more rapidly, while the speed of the destruction of the primary graft was apparently not affected Indeed, some primary grafts survived for more than twenty days after the secondary ones had disintegrated Although our data are relatively few, the results suggest that the grafts' one set of host-specific chromosomes protects them in a measured amount of time against the antibodies of the host

With regard to sex, Eichwald *et al* (1957) had shown that skin transplants would not survive in certain inbred strains of rats if the donor was a male and the recipient a female The failure of their transplants to take may be attributed to a histocompatibility gene located on the Y chromosome of the male From our experiments we failed to obtain any evidence of sex linkage In our transplants all hosts and donors were chosen completely at random In platyfish sex is recognizable by sight only beginning about 90 days after hatching Since normally the fish of strains 30 and 163 differentiate into 50 per cent males and 50 per cent females, approximately one fourth of our transplants must have been from male to male, one fourth from male to female,

one fourth from female to male, and one fourth from female to female The uniform success of all our intrastrain grafts and grafts into the $F_1$ hosts precludes any effect of sex upon the fate of a transplant in these strains

With regard to age, tolerance to homografts has been experimentally induced in mice by Billingham *et al* (1953), in rats by Woodruff and Simpson (1955), and in the chick by Cannon and Longmire (1952) by grafting cells from the donor into a host at an early developmental stage or within a short time after hatching or birth In an earlier paper, however, Kallman and Gordon (1957) reported that homografts of fins into hosts less than 2 days old were all rejected within 9 days Recently Humm *et al* (1957) used swordtail, platyfish, and platyfish-swordtail hybrid embryos obtained by cesarean section They transplanted bits of melanoma tissue of swordtail-platyfish hybrids into the extraembryonic membrane of these hybrids at the developmental stage 20 This is the stage equivalent to the eleventh day after first cleavage of the egg and 11 days before they would normally have been born, as determined by Tavolga (1949) Humm *et al* found that in most embryos the melanoma cells initially grew and spread into the surrounding area, in some the cells invaded the embryo proper About 60 per cent of the embryos died within 7 days after receiving the transplant, following a stasis and engorgement of the blood vessels of the yolk sac This circulatory failure they judged to be an immunological response of the host toward the graft A small number of embryos died after the tumor cells had invaded their pericardia and impeded the action of their hearts physically Some of the remaining embryos retained the transplant for 40 to 70 days, but eventually all the transplants disintegrated Thus, tolerance to homografts was not induced in these fish embryos

## Summary


(1) In order to study the genetic aspect of transplantation in fish, anal, caudal, and dorsal fins were transplanted among 2 highly inbred strains of *Xiphophorus maculatus* and their interstrain hybrids

(2) Sixty-six intrastrain grafts were made, and all were permanently accepted

(3) One hundred interstrain grafts were made, but all were rejected

(4) Sixty-one fin grafts obtained from the parent strains and transplanted into their $F_1$ hybrids were successful Thirty-two grafts transplanted among the $F_1$ hybrids were also successful On the other hand, 60 fins obtained from the $F_1$ hybrids and transplanted into members of the parent strains were all rejected

(5) The survival time of the interstrain grafts varied from 5 to 15 days The survival time of the $F_1$ grafts which were transplanted into $P_1$ hosts varied from 7 to 67 days All secondary grafts were rejected between 2 and 7 days

(6) The survival time of rejected grafts also reflects, in a measure, the degree of genetic relationship between donor and host

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REFERENCES

BILLINGHAM, R. E, L BRENT & P B MEDAWAR 1953 Actively acquired tolerance of foreign cells Nature 172 603–606

CANNON, J A. & W P LONGMIRE 1952 Studies on successful skin homografts in the chicken Ann Surg 135 60–68

EICHWALD, E J, C R. SILMSER & N WHEELER 1957 The genetics of skin grafting Ann N Y Acad Sci 64(5) 737–740

GOODRICH, H B & R. NICHOLS 1933 Scale transplantation in the goldfish, *Carassius auratus* I Effects on chromatophores II Tissue reactions Biol Bull 66 253–265

GORDON, M 1950 Fishes as laboratory animals *In* Care and Breeding of Laboratory Animals 14 345–449 E J Farris, Ed Wiley & Sons New York, N Y

HELD, E E 1951 Morphogenesis of the gonopodium and fin regeneration in poeciliid fishes Thesis Univ Calif Los Angeles, Calif

HILDEMANN, W H 1957 Scale homotransplantation in goldfish (*Carassius auratus*) Ann N Y Acad Sci 64(5) 775–791

HUMM, D G, E E CLARK & J H HUMM. 1957 Transplantation of melanomas from platyfish-swordtail hybrids into embryos of swordtails, platyfish and their hybrids Brit J Exptl Biol. 34 518–528

KALLMAN, K D & M GORDON 1957 Transplantation of fins in xiphophorin fishes Ann N Y Acad Sci 71(3) 305–320

SNELL, G D 1953 The genetics of transplantation J Natl Cancer Inst 14 691–700

TAVOLGA W N 1949 Embryonic development of the platyfish (*Platypoecilus*), the swordtail (*Xiphophorus*), and their hybrids Bull Am Museum Nat Hist 94 161–229

WOODRUFF, M F A & L O SIMPSON 1955 Induction of tolerance to skin homografts in rats by injection of cells from the prospective donor soon after birth Brit J Exptl Pathol 36 494–499

# GENETICS OF TISSUE SPECIFICITY*

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## *Introduction*

Throughout most of its brief history, immunogenetics has been dominated by a striking theoretical concept, applying equally well to the genetics of erythrocyte antigens and to those responsible for transplantation specificity [1-3] In both instances, the presence of an antigenic specificity is usually dominant over its absence, different antigenic specificities associated with allelic genes are simultaneously present in the heterozygote, alleles rarely interact in the heterozygote to produce new antigenic specificities, and the antigenic effect of a given gene seems rarely to be altered by genes at other loci This combination of circumstances, apparently demonstrating a lack of genic interaction in the determination of antigenic specificity, has led to a conclusion which, for erythrocytic antigens, has been stated by Irwin[4] as follows

"The sequence of events between the genes and the cellular antigens which are produced appears to be relatively direct That is, if there is a series of reactions between the gene and the end product, the cellular antigen, there are no known instances in which the chain of reactions may be interrupted, by another gene or otherwise "

More generally, Wright[5] has suggested that

"The likelihood that the specificity of antigens reflects that of genes more directly than does any other character makes progress in this field of special interest in physiological genetics "

In the field of transplantation genetics the facts summarized above have emerged from the pioneering work of Little and his associates,[6] extended by the brilliant work of Snell and his collaborators,[7] and from the immunological contributions of Gorer[8] and, more recently, of Billingham *et al* [9] Little was concerned with establishing the Mendelian nature of the genetic influences determining susceptibility and nonsusceptibility to the growth of transplanted tissue It is of interest to note that even in his most recent publications[10] Little does not refer to the apparent lack of genic interaction in the determination of antigenic specificity, he restricts himself to the demonstration that Mendelian genes are in large part responsible for transplantation specificity, and that many such loci are involved The idea that the genes at each of these loci produce their antigenic effects without interaction grew with expansion of the work with erythrocytic antigens by the Wisconsin group (Irwin *et al* ),[3, 4] although it apparently was formulated by both Wright[5] and Haldane[2] even before that time In any event, it now seems to be implicit in the minds of most transplantation workers [11]

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For almost ten years we have been engaged in investigations of gene-antigen relationships in *Drosophila melanogaster* In every case studied to date, apparent instances of genic interaction have been encountered It is the purpose of this paper to review this work, to present some new evidence, and to discuss its implications for the genetical theory of transplantation specificity

*Genetic Methods*

In all of this work strict adherence to the use of coisogenic stocks has been observed Pertinent details regarding the derivation of these stocks will be given in succeeding sections The method is not different in principal from that involved in the use of mice of inbred and isogenic resistant (IR) stocks,[12] except that the approach to isogenicity and coisogenicity is more readily achieved in *Drosophila* than in the mouse The purpose is to make available numerous individuals of the same genotype (isogenic), and stocks differing only with respect to known, identifiable loci (coisogenic) Antigenic differences among the former would then be attributable to environmental factors, while antigenic differences among the latter could be attributed strictly to the genic substitutions involved Environmental conditions have not been narrowly controlled in our work, but no systematic search has been made for environmental effects and none have been observed

Unfortunately, isogenicity is an ideal that can be approached but never reached in practice In the mouse it can be approached only through inbreeding, which even after many generations does not eliminate residual variability with certainty[13, 14] This uncertainty has had its occasional consequences in transplantation work[15] In *Drosophila*, isogenicity can be approached more rapidly by the use of dominant markers and inversions suppressing crossing-over,[16] but even here complete success cannot be assured[17] Even if complete isogenicity were attainable, it would be destroyed quickly by uncontrollable spontaneous mutations Instances of this sort with antigenic consequences have been encountered in mouse immunogenetics,[15] and we shall discuss a case that occurred in our own work In spite of these qualifications the methods that we have used are among the best available for the achievement of isogenicity, and there is no evidence that possible failure to achieve this ideal has in any way affected our results

Many of the same qualifications apply to the attainment of coisogenicity Spontaneous mutation in an isogenic stock is the most reliable source of a coisogenic genotype, since the frequency of such mutation is sufficiently low as to render the simultaneous change of two loci improbable Nevertheless, closely linked loci (pseudoalleles) sometimes exhibit a tendency to mutate simultaneously,[18, 19] that is, to comprise a single mutational unit, and the uncontrollability of the process makes it an impracticable one As an alternative, mutations may be induced on an isogenic background by means of radiation or some other mutagenic agent The probability of simultaneous change at two loci is appreciably higher in this instance than in the case of simultaneous mutation, but this difficulty can be overcome except for closely linked loci Insofar as I know this method has not been used in mouse im-

munogenetics, but we have used it in our work with *Drosophila*.[17] Its applicability is limited by the difficulty of obtaining particular desired mutations at will. A method that accomplishes this aim but is less reliable with regard to coisogenicity is that of the insertion of a given gene into an isogenic background by means of crossing-over. This is the basis of Snell's methods for the derivation of IR stocks. The difficulty, as has been noted by Snell[12] and treated quantitatively by Fisher[20] is that a chromosomal segment of uncontrollable length on both sides of the locus in question is inserted along with the desired gene. The length of this segment can be controlled and reduced by the use of closely linked markers, a potentiality of which we have availed ourselves in *Drosophila*.[21, 22] Finally, even if complete coisogenicity of two stocks could be attained, they would quickly diverge because of spontaneous mutation. Where practicable, we have taken steps to avoid such divergence.[23]

*Immunological Methods*

As a source of antigens, large numbers of flies of a given genotype are collected from numerous cultures. Standard corn meal medium is used, enriched with brewer's yeast but not seeded with living yeast. Flies are collected only from cultures not visibly contaminated with microorganisms. Special tests have demonstrated that flies grown on bacteriologically sterile medium possess the same antigenic specificities as those grown without precautions other than the preceeding.[24] After collection, the flies are starved for 12 to 24 hours to eliminate food from the gut, are quickly frozen at −70° C., and are lyophilized. When antigenic preparations are needed for immunization or for serological tests, the lyophilized flies are homogenized with an all-glass homogenizer in cold saline (0.85 per cent NaCl + 0.005 M phosphate, *p*H 7.4), in a ratio of 2 gm. of lyophilized flies per 100 ml. of saline. Such homogenates contain 1.57 mg. total N per ml.

The whole homogenate is used for the immunization of rabbits via the intraperitoneal route. Experience has proved that this is the most effective means of immunization, the particulate matter in the homogenate probably serving as a sort of natural adjuvant. A total of 22 ml. of homogenate is administered in 4 doses on alternate days, followed by an additional 8 ml. after 1 week if preliminary titration of the serum makes this desirable. The rabbits are exsanguinated 1 week after the last dose. This immunization schedule yields low-titered but highly specific antisera. One of the difficulties encountered is the differential response of individual rabbits to the complex antigenic preparations, necessitating in some cases the immunization of numerous animals with the antigens extracted from flies of a particular genotype. The existence of all antigenic components to be described has been confirmed by the use of several to many antisera.

Serological tests are performed with the clear supernates obtained by centrifugation of the homogenates. These contain 0.54 mg. of protein N per ml., precipitable by trichloracetic acid (TCA). Precipitin techniques have been used exclusively, early work having demonstrated the unsuitability of complement fixation with whole homogenates.[25] In early work, a semi-

quantitative method involving absorption and precipitin-ring techniques was used for the identification of antigenic components specific to particular genotypes[17,21,22]. This method was difficult at best, requiring careful control and capable of demonstrating only a limited number of specific components, its use was therefore restricted to the demonstration of qualitative differences, that is, only those remaining after exhaustive absorption. More recently, agar-diffusion techniques have been used to good advantage[23,26,27].

*Interaction Between Nonalleles*

Application of the methods described above disclosed interaction between nonallelic loci in the very first case examined[17]. For purposes of the analysis an isogenic wild stock was derived by the use of dominant markers and inversions. Two separate mutations were then induced at widely separated loci on the X chromosome by means of X rays, one at the vermilion locus ($v^{48a}$) and one at the ruby locus ($rb^{48a}$). Each of these was incorporated into a mutant stock coisogenic with the wild stock, by replacing all irradiated chromosomes except the appropriate X with chromosomes from the wild stock. A double mutant stock, ruby-vermilion, was then derived by mating the single mutants with each other and extracting a cross-over X chromosome. The genotypes of the 4 stocks resulting from this derivation are represented diagrammatically in TABLE 1. As may be seen, the ruby stock differs from wild with respect to the *rb* locus, the vermilion stock with respect to the *v* locus, and the double mutant stock with respect to both loci. Within the limits discussed above, the residual genotype is the same in all four stocks.

Analysis of antisera to the antigens extracted from each of the four stocks was performed by means of reciprocal absorptions and precipitin-ring tests with the antigenic preparations of all four stocks. Thus, for example, unabsorbed antiwild serum reacted with equal titer with the antigens extracted from all four stocks, giving evidence of antigenic components common to all four stocks, presumably the products of loci in the residual genotype. Absorption with ruby extract, even in the region of great antigen excess, failed to inhibit the reaction of such antiserum with the extract of wild flies, although its reactivity with the antigens of ruby, vermilion, and ruby-vermilion was thereby removed. It could therefore be concluded that wild possesses an antigenic component (antigen 1) not present in any of the three mutant genotypes, a conclusion that was confirmed by absorptions with vermilion and ruby-vermilion antigenic preparations. In a similar manner, a second specific

TABLE 1
STOCKS USED IN DEMONSTRATION OF NONALLELIC INTERACTION

| Stocks | Genotypes | | |
|---|---|---|---|
| Wild | $rb^{+}$ | $v^{+}$ | residual |
| Ruby | $rb^{48a}$ | $v^{+}$ | residual |
| Vermilion | $rb^{+}$ | $v^{48a}$ | residual |
| Ruby-vermilion | $rb^{48a}$ | $v^{48a}$ | residual |

TABLE 2
DISTRIBUTION OF ANTIGENIC COMPONENTS

| Stocks | Antigenic components | | |
|---|---|---|---|
| | 1 | 2 | Residual |
| Wild | + | − | + |
| Ruby | − | − | + |
| Vermilion | − | + | + |
| Ruby-vermilion | − | + | + |

component (antigen 2) could be demonstrated in vermilion and ruby-vermilion The distribution of these components is summarized in TABLE 2

Correlation of the distribution of these antigens with the genotypes represented in TABLE 1 immediately discloses that antigen 1 is found only in the presence of both $rb^+$ and $v^+$ If a mutant allele is substituted for either of these wild alleles, or for both, the antigen is not produced This antigen must therefore be considered a product of the interaction of the two wild alleles Furthermore, since the residual genotypes are the same in all four stocks, it cannot be excluded that other loci are also involved in this interaction

The presence of antigen 2 in both stocks possessing the mutant allele of vermilion, regardless of the genotype for the *rb* locus, suggests that this antigen is the product of $v^{48a}$ without interaction with $rb^+$ or $rb^{48a}$ As with antigen 1, however, the involvement of one or more residual loci in its production cannot be excluded

To exclude the remote possibility that undetected mutations at X-chromosomal loci other than *rb* and *v* were responsible for the observed antigenic differences between the stocks, a new series of stocks were derived[28] by mating the original ruby-vermilion with the original wild and extracting, by means of crossing-over, new ruby and vermilion single-mutant stocks These were then mated and new wild and ruby-vermilion stocks were established, again by means of crossing-over The derivation of this new series of stocks therefore provided at least two opportunities for the separation, by crossing-over, of undetected mutations from the established alleles at the *rb* and *v* loci Antigenic analysis of these stocks disclosed the presence of antigens 1 and 2 in a distribution identical to that exhibited in the original series The distribution could therefore be attributed, with even greater confidence, to the effects of genic substitution at the *rb* and *v* loci

To elucidate the role of the residual genotype in the production of these antigens, each of the original coisogenic series of stocks was outcrossed to an unrelated wild stock, and new wild, ruby, vermilion, and ruby-vermilion stocks were extracted [28] Due to the manner of its derivation, each of these new stocks possessed the same genotype at the *rb* and *v* loci as the corresponding original stock (wild, $rb^+ v^+$, ruby, $rb^{48a} v^+$, vermilion, $rb^+ v^{48a}$, ruby-vermilion, $rb^{48a} v^{48a}$), but on a different genetic background Upon analysis, it was found that antigen 1 was not present in any of these stocks Since the new wild

stock possesses the identical $rb^+$ and $v^+$ genes as the original, the absence of antigen 1 must be attributed to the change in the residual genotype Thus, at least one additional locus is involved, together with $rb^+$ and $v^+$, in the interaction producing antigen 1 Antigen 2, on the other hand, was present in both the new vermilion and ruby-vermilion stocks Its unique association with $v^{48a}$ was thus more firmly established, and no genic interaction in its formation could be demonstrated

At least three loci, two of which have been identified, are therefore involved in the production of antigen 1 Two additional circumstances strongly suggest that the actual number of genes involved is larger than three In the first place, antigen 1 was found to be absent from still another unrelated wild stock (Oregon R) Even more suggestive was the disappearance of antigen 1 from the original wild stock about three years after its derivation No change of $rb^+$ or $v^+$ had occurred during that period, but if a fairly sizable number of additional loci were involved in the production of antigen 1, mutation of at least one of these could readily occur, resulting in the disappearance of the antigen from the stock

With respect to antigen 2, it should be amply evident that the failure to demonstrate genic interaction does not exclude that possibility The nature of the methodology of genetic analysis is such that a gene can be identified and its effects demonstrated only if there are available at least two allelic alternatives in the material at hand It is accordingly possible that additional loci are concerned with the production of antigen 2 but were not detected because they were not changed by substitutions of residual genotype made in the derivation of the last series of stocks To put this possibility in another way, the pertinent residual loci may have been the same in the original isogenic stock and in the unrelated wild stock from which the new genetic background was derived Considerations such as these serve the purpose of demonstrating that while positive evidence for genic interaction is methodologically attainable the absence of interaction is difficult to establish Recognition of this methodological limitation will be important for our subsequent evaluation of transplantation immunogenetics

Before the disappearance of antigen 1 from the original isogenic stock, considerable progress was made in its isolation and characterization[29] The antigen was demonstrably nondialyzable, its absorption spectrum was that of a typical protein, and it was positive for the usual protein tests It exhibited anodic electrophoretic mobility at *p*H 7 0, was water soluble, was inactivated by NaCl concentrations above 0 5 M, and was precipitated by 44 4 per cent ethanol at −5° C, *p*H 7 0

While data of this sort do not yield any direct information regarding the biosynthetic mechanisms by means of which genic interaction in the production of antigens might be accomplished, the distribution of antigens among the stocks may be used in the construction of a scheme such as that contained in FIGURE 1[23] Such a scheme is purely formal, of course, and should not be taken as literally representing the processes involved in the production of the antigens A number of other schemata fitting the data equally well might be

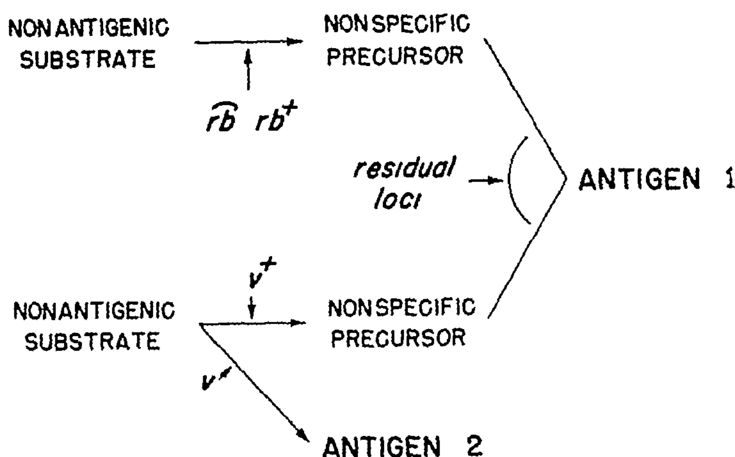



FIGURE 1 Formal scheme of antigenic distribution

proposed, but all exhibit a common characteristic The various steps proposed in each fall into two general categories a series of preliminary processes leading to the production of nonspecific precursors, and a second stage during which final specificity is conferred upon the antigen Such a point of view regarding the genetic control of protein synthesis has been of heuristic value in subsequent immunogenetic work with *Drosophila* (described below), has been strikingly illustrated in separate work on the genetic control of tyrosinase synthesis and specificity in *Neurospora*,[30, 31] and resembles very closely the suggestions made by Haurowitz on the basis of purely immunological and biochemical considerations [32]

*Interaction Between Pseudoalleles*

Shortly after completion of the work just described, it was demonstrated by M M Green that certain of the vermilion mutants are pseudoalleles rather than alleles [33] Pseudoallelic mutants occupy separable but closely linked loci, so that their mode of transmission resembles that of nonallelic mutants, that is, they exhibit a low order of recombination resulting from crossing-over Their similarity of phenotypic effects and the interactions they exhibit in heterozygotes are, however, reminiscent of the physiological relationships characteristic of true alleles

All of the vermilion mutants in *Drosophila* produce similar effects on eye color, resulting from their inability to support the conversion of tryptophane into formylkynurenine, which serves as the precursor of kynurenine in the synthesis of brown eye pigment In an elegant experiment, Green[33] demonstrated a low frequency of crossing-over between the mutants $v^1$ and $v^{36f}$, thus disclosing that they are mutants at two closely linked loci bearing the following linkage relationships to the raspberry and minature loci on the X chromosome

$$ras\ 0\ 2\ v^1\ v^{36f}\ 3\ 1\ m$$

Inherent interest in the problems posed by the phenomenon of pseudoallelism

for the theory of the gene, and our previous work with $v^{48a}$, led us to an investigation of the immunogenetics of the vermilion pseudoalleles [22]

The three mutants $v^1$, $v^{36f}$, and $v^{48a}$ were used for this purpose Extensive genetic tests, involving examination of over 150,000 progeny from critical matings, disclosed crossing-over between $v^{36f}$ and $v^{48a}$ but not between $v^1$ and $v^{48a}$ Therefore, $v^{48a}$ must be an allele of $v^1$, that is, another mutant at that locus The antigenic effects of three alleles at the $v^1$ locus could therefore be observed wild ($+^1$), $v^1$, and $v^{48a}$ Two alleles at the $v^{36f}$ locus were available wild ($+^{36f}$), and $v^{36f}$ The pseudoallelism of $v^1$ and $v^{36f}$ made it possible to study the double mutant $v^1\ v^{36f}$ as well as the single mutants

Coisogenic stocks differing with respect to these mutants were derived by inserting the vermilion segments of each into the X chromosome of an isogenic wild stock (Oregon R) This was accomplished by means of crossing-over between *ras* and the $v^1$ locus on the left, and between the $v^{36f}$ locus and *m* on the right Each of these stocks possessed the Y chromosome and autosomes of isogenic Oregon R origin Within the usual qualifications applying to coisogenicity, they should differ only with respect to the vermilion loci, except that their X chromosomes may differ also with respect to a segment no longer the 0 2 cross-over units to the left and 3 1 units to the right of the vermilions The following genotypes were then subjected to antigenic analysis wild (Oregon R), the three single mutants ($v^1$, $v^{36f}$, and $v^{48a}$), the double mutant ($v^1\ v^{36f}$), and the *cis* and *trans* double heterozygotes for $v^1$ and $v^{36f}$ ($v^1\ v^{36f}/+$ and $v^1/v^{36f}$) Complete representation of these genotypes is given in TABLE 3

In addition to residual antigens present alike in all genotypes, methods similar to those used in the previous case disclosed qualitative differences with respect to three specific components (TABLE 4), thus demonstrating a resolution of our old antigen 2 The first observation to be made about these results is one concerned with the peculiarities of pseudoallelism the antigenic effects of all three mutants are similar, but alleles ($v^1$ and $v^{48a}$) are if anything

TABLE 3
GENOTYPES USED IN ANALYSIS OF VERMILION PSEUDOALLELES

| Genotypes | Complete representation | | |
|---|---|---|---|
| Oregon R | $+^1$ | $+^{36f}$ | residual |
| $v^1$ | $v^1$ | $+^{36f}$ | residual |
| $v^{36f}$ | $+^1$ | $v^{36f}$ | residual |
| $v^{48a}$ | $v^{48a}$ | $+^{36f}$ | residual |
| $v^1\ v^{36f}$ | $v^1$ | $v^{36f}$ | residual |
| $v^1\ v^{36f}/+$ | $\frac{v^1}{+^1}$ | $\frac{v^{36f}}{+^{36f}}$ | residual |
| $v^1/v^{36f}$ | $\frac{v^1}{+^1}$ | $\frac{+^{36f}}{v^{36f}}$ | residual |

TABLE 4

ANTIGENIC COMPONENTS AFFECTED BY VERMILION PSEUDOALLELES

| Genotypes | Antigenic components | | | |
|---|---|---|---|---|
| | V-1 | V-2 | V-3 | Residual |
| Oregon R | − | − | Haptenic | + |
| $v^1$ | + | − | − | + |
| $v^{36f}$ | + | + | − | + |
| $v^{48a}$ | + | + | + | + |
| $v^1$ $v^{36f}$ | + | + | + | + |
| $v^1$ $v^{36f}$/+ | + | + | − | + |
| $v^1$ $v^{36f}$ | + | + | − | + |

less similar in effect than pseudoalleles ($v^{48a}$ and $v^{36f}$) Beyond this, it is of great interest to note that a change at but one of the two pseudoallelic loci may result in changes with respect to more than one antigenic specificity Thus, $v^{36f}$ is associated with two antigenic components (V-1 and V-2), while $v^{48a}$ is associated with three (V-1, V-2, and V-3) This observation has an immediate bearing on the nature of the histocompatibility-2 (H-2) locus in the mouse, each allele of which is associated with more than one antigen [34] On the supposition that each specificity is the product of a different locus, it has been suggested that H-2 is really a series of pseudoallelic loci In the present case, which is one of demonstrated pseudoallelism, alteration of the individual loci still results in multiple antigenic effects Thus, while the demonstration of crossing-over between different H-2 alleles would not come as a surprise, the conclusion that they are pseudoallelic does not necessarily follow from the complexity of their antigenic effects

The most striking feature of the antigenic effects of the vermilion pseudoalleles concerns the distribution of antigen V-3 This antigen is present in $v^{48a}$ and $v^1$ $v^{36f}$ in complete form, that is, in a form capable of inducing antibody formation and of uniting with the antibody to form visible precipitate In these genotypes it is nondialyzable, is precipitated by protein precipitants (TCA and alcohol), and is denatured by heat It is not present in $v^1$, or $v^{36f}$, or in their double heterozygotes extracts of these genotypes lack the power of invoking the formation of the V-3 antibody and of inhibiting it upon absorption of anti-$v^{48a}$ or anti-$v^1$ $v^{36f}$ sera Thus, the double mutant ($v^1$ $v^{36f}$) is capable of producing an antigenic component which is not produced by either of its mutant genes separately ($v^1$ or $v^{36f}$), but which is produced by a different mutant ($v^{48a}$) at one of the two loci In the double mutant the antigen must be the product of interaction of $v^1$ and $v^{36f}$ It therefore represents an additional case of genic interaction in antigen formation—a case of interaction between pseudoalleles

Insight into the mechanism of this interaction is afforded by the observation that V-3 is present in Oregon R in what appears to be a haptenic form Oregon R extracts are incapable of inducing the formation of the V-3 antibody, but will inhibit it upon absorption of anti-$v^{48a}$ or anti-$v^1$ $v^{36f}$ sera without,

however, the formation of precipitate In contrast to the physical properties that it exhibits in $v^{48a}$ and $v^{1}$ $v^{36f}$, V-3 in Oregon R is heat stable although non-dialyzable Genic substitution at the pseudoallelic vermilion loci therefore has two distinct effects on V-3, one having to do with the production of the specificity itself and the other determining whether the specificity is present in haptenic form or associated with protein The congruence of this situation to the two-stage hypothesis of protein synthesis formulated in the preceeding section is immediately apparent

It would appear that the simplest way of regarding the mechanisms of V-3 synthesis is to suppose that the vermilion loci interact to produce the specificity-determining hapten, while the protein moiety to which it may be coupled is produced by other loci in the residual genotype Together with Chovnick and Lefkowitz, we have elsewhere discussed evidence that indicates that a whole pseudoallelic segment containing more than one locus may act as a unitary physiological agent [35] In the present instance, the basis of genic interaction in the production of V-3 specificity would appear to reside in the integrated action of the whole vermilion segment, consisting of two loci, as a physiological unit

*Interaction Between Alleles*

A classic case in the elucidation of the phenomenon of pseudoallelism in *Drosophila* is that of the lozenge mutants [36] Three closely linked loci on the X chromosome are involved in this pseudoallelic series, to which are now assigned the symbols *spe*, *lz*, and *gly* [37] Their linkage relationships with respect to the singed (*sn*) and vermilion (*v*) loci are as follows

$$sn \; 6\;7 \; spe \; 083 \; lz \; 057 \; gly \; 5\;3 \; v$$

In an investigation paralleling the previous one,[21] the antigenic effects of three lozenge mutants were studied $spe^{BS}$ (formerly $lz^{BS}$), $lz^{46}$, and $gly^{1}$ (formerly $lz^{g}$) Coisogenic stocks differing with respect to these mutants were constructed by methods similar to those used in the preceeding case, that is, by the insertion through crossing-over of the lozenge mutants into the X chromosome of the isogenic Oregon R stock Qualification of coisogenicity in this case arose from the fact that, aside from differences with respect to the lozenge loci, the X chromosomes of the stocks may have differed with respect to a segment no more than 6 7 cross-over units long to the left and 5 3 units long to the right of the lozenges The genotypes that were examined are given in TABLE 5

Antigenic analysis was performed as in the preceeding cases, and the results are given in TABLE 6 In many respects, these results resemble those obtained with the vermilion pseudoalleles The similarity of the antigenic effects of pseudoallelic mutants is once again displayed, as is the association of more than one antigen with a single gene Beyond this, the appearance of a distinctive antigenic component, L-3, in the heterozygous genotypes ($spe^{BS}$ $lz^{46}/+$ and $spe^{BS}/lz^{46}$) is of particular interest Neither of the two mutants alone, nor their respective wild alleles, produce this antigen The antigen

TABLE 5
GENOTYPES USED IN ANALYSIS OF LOZENGE PSEUDOALLELES

| Genotypes | Complete representation | | | |
|---|---|---|---|---|
| Oregon R | $spe^+$ | $lz^+$ | $gly^+$ | residual |
| $spe^{BS}$ | $spe^{BS}$ | $lz^+$ | $gly^+$ | residual |
| $lz^{46}$ | $spe^+$ | $lz^{46}$ | $gly^+$ | residual |
| $gly^1$ | $spe^+$ | $lz^+$ | $gly^1$ | residual |
| $spe^{BS}$ $lz^{46}/+$ | $\frac{spe^{BS}}{spe^+}$ | $\frac{lz^{46}}{lz^+}$ | $\frac{gly^+}{gly^+}$ | residual |
| $spe^{BS}/lz^{46}$ | $\frac{spe^{BS}}{spe^+}$ | $\frac{lz^+}{lz^{46}}$ | $\frac{gly^+}{gly^+}$ | residual |

TABLE 6
ANTIGENIC COMPONENTS AFFECTED BY LOZENGE PSEUDOALLELES

| Genotypes | Antigenic components | | | |
|---|---|---|---|---|
| | L-1 | L-2 | L-3 | Residual |
| Oregon R | − | − | − | + |
| $spe^{BS}$ | + | + | − | + |
| $lz^{46}$ | + | + | − | + |
| $gly^1$ | + | − | − | + |
| $spe^{BS}$ $lz^{46}/+$ | − | − | + | + |
| $spe^{BS}/lz^{46}$ | − | − | + | + |

must be the product of interaction between $spe^{BS}$ and its wild allele ($spe^+$), or between $lz^{46}$ and its wild allele ($lz^+$), or both As such, it represents a third case of genic interaction in the production of antigens, this time a case of interallelic interaction

*Antigenic Effects of Changes in Chromosome Balance*

The most recent case studied has concerned itself with antigenic differences between males and females and the role of the X and Y chromosomes in their determination Since normal males and females differ both with respect to the presence (in males) and absence (in females) of the Y chromosome and with respect to number of X chromosomes (one in males, two in females), a special series of stocks is necessary for elucidation of the role of each [33]

Two coisogenic stocks have been used for this purpose (TABLE 7) One of these is the isogenic Oregon R stock used in previous studies Oregon R

however, the formation of precipitate In contrast to the physical properties that it exhibits in $v^{48a}$ and $v^{1}$ $v^{36f}$, V-3 in Oregon R is heat stable although non-dialyzable Genic substitution at the pseudoallelic vermilion loci therefore has two distinct effects on V-3, one having to do with the production of the specificity itself and the other determining whether the specificity is present in haptenic form or associated with protein The congruence of this situation to the two-stage hypothesis of protein synthesis formulated in the preceeding section is immediately apparent

It would appear that the simplest way of regarding the mechanisms of V-3 synthesis is to suppose that the vermilion loci interact to produce the specificity-determining hapten, while the protein moiety to which it may be coupled is produced by other loci in the residual genotype Together with Chovnick and Lefkowitz, we have elsewhere discussed evidence that indicates that a whole pseudoallelic segment containing more than one locus may act as a unitary physiological agent [35] In the present instance, the basis of genic interaction in the production of V-3 specificity would appear to reside in the integrated action of the whole vermilion segment, consisting of two loci, as a physiological unit

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*sn* 6 7 *spe* 083 *lz* 057 *gly* 5 3 *v*

In an investigation paralleling the previous one,[21] the antigenic effects of three lozenge mutants were studied $spe^{BS}$ (formerly $lz^{BS}$), $lz^{46}$, and $gly^{1}$ (formerly $lz^{g}$) Coisogenic stocks differing with respect to these mutants were constructed by methods similar to those used in the preceeding case, that is, by the insertion through crossing-over of the lozenge mutants into the X chromosome of the isogenic Oregon R stock Qualification of coisogenicity in this case arose from the fact that, aside from differences with respect to the lozenge loci, the X chromosomes of the stocks may have differed with respect to a segment no more than 6 7 cross-over units long to the left and 5 3 units long to the right of the lozenges The genotypes that were examined are given in TABLE 5

Antigenic analysis was performed as in the preceeding cases, and the results are given in TABLE 6 In many respects, these results resemble those obtained with the vermilion pseudoalleles The similarity of the antigenic effects of pseudoallelic mutants is once again displayed, as is the association of more than one antigen with a single gene Beyond this, the appearance of a distinctive antigenic component, L-3, in the heterozygous genotypes ($spe^{BS}$ $lz^{46}/+$ and $spe^{BS}/lz^{46}$) is of particular interest Neither of the two mutants alone, nor their respective wild alleles, produce this antigen The antigen

The Ouchterlony technique discloses a minimum of 7 antigenic components in all 4 genotypes (residual), 2 components specific to Oregon R and YA females (♀-1 and ♀-2), and 2 components specific to Oregon R and YA males (♂-1 and ♂-2)[23] Each component is represented by a distinct line of precipitate in the agar of the Ouchterlony plates By means of the Bjorklund technique the lines representing residual components are inhibited by antigen extracts of all 4 genotypes The 2 female-specific components are inhibited by extracts of either female genotype but neither male genotype The 2 male-specific components are inhibited by extracts of either male genotype but neither female genotype The failure of antigenic extracts to inhibit is observed even when their concentration is increased fiftyfold to one-hundredfold by dialysis and lyophilization, and it may be demonstrated also by absorption of the antisera prior to performance of the Ouchterlony tests The differences between males and females are therefore qualitative in nature

Since these differences are not associated with the presence or absence of the Y chromosome they must be attributed to the difference between the 2 types of males and the 2 types of females with respect to X-chromosomal dosage In the presence of two X chromosomes the female components are produced, while the male components are produced in the presence of one X chromosome This is the first case of which we are aware in which a change in gene dosage has been observed to result in a qualitative shift in antigenic specificity It is not unlikely that the mechanism responsible for this shift involves the balance of X chromosomes with respect to autosomes, as in sex determination In any event, the hypothesis of dominant histocompatibility genes producing their effects without interaction cannot account for such a shift, genic interaction must be invoked

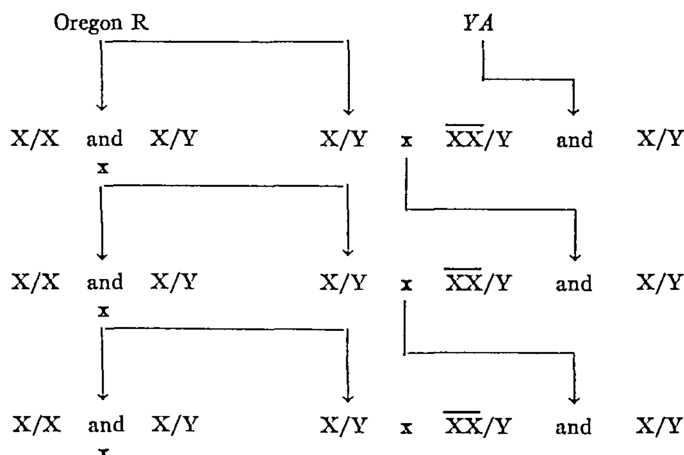
These observations are particularly pertinent for the interpretation of the discovery by Eichwald and Silmser[39] that male transplants are rejected by female hosts in certain inbred lines of mice Since females lack a Y chromosome, it has been suggested that Y-borne histocompatibility genes are responsible for this phenomenon[16, 40] This suggestion, however, follows only from the assumption that all histocompatibility genes are dominant and produce their antigenic effects without interaction Antigens such as our ♂-1 and ♂-2 could account for the observation of Eichwald and Silmser The clear distinction between Y-chromosomal effect and X-chromosomal dosage effect requires an examination of XXY females or XO males, neither of which are available in mice

### *Antigenic Effects of the Y Chromosome*

An additional antigenic difference has been disclosed by the foregoing analysis, serving to distinguish between the Oregon R stock on the one hand and the YA stock on the other[27] The antigen involved has been named Y-1 Antisera to the antigenic extracts of any of the four genotypes listed in TABLE 7 contain the antibody that serves to identify this antigen in Ouchterlony tests The corresponding precipitate line is formed, however, only upon reaction of the antibody with the antigenic extracts of Oregon R males or females, no

TABLE 7

MATING SYSTEM USED IN MAINTAINING OREGON R AND YA STOCKS




females have two X chromosomes (X/X), while Oregon R males have one X and a Y (X/Y) The other stock is called YA Its males are X/Y, and are genotypically identical with Oregon R males YA females, on the other hand, possess attached X chromosomes, and consequently have a Y chromosome in addition to their two X chromosomes ($\overline{XX}$/Y) In each generation the YA stock is maintained by mating YA females with Oregon R males, thus preventing genotypic divergence of the two stocks Since the stocks are coisogenic, antigenic differences distinguishing the two types of females from the two types of males are to be attributed to the difference in X-chromosome dosage (two in females, one in males) Antigenic differences distinguishing Oregon R females on the one hand from Oregon R males, YA females, and YA males on the other would be attributable to the presence of the Y chromosome in the latter genotypes and its absence in the former

In this case, antigenic analysis has been performed by means of the agar-diffusion technique of Ouchterlony and the inhibition technique of Bjorklund Details are provided elsewhere [23, 27] The results are summarized in TABLE 8

TABLE 8

ANTIGENIC COMPONENTS IN OREGON R AND YA STOCKS

| Antigenic Components | Oregon R | | YA | |
|---|---|---|---|---|
| | X/X | X/Y | $\overline{XX}$/Y | X/Y |
| ♀-1 | + | − | + | − |
| ♀-2 | + | − | + | − |
| ♂-1 | − | + | − | + |
| ♂-2 | − | + | − | + |
| Y-1 | Complete | Complete | Incomplete | Incomplete |
| Residual | + | + | + | + |

TABLE 9

MATING SYSTEM USED TO REMOVE Y CHROMOSOME FROM YA CYTOPLASM

$\overline{XX}$/Y x X Y/O

↓

$\overline{XX}$/O x X Y/O

↓

$\overline{XX}$/O and X Y/O

must be concluded that the form of the antigen depends upon the presence or absence of the Y chromosome in the oocyte of the female that produces the individuals being examined

For purposes of clarity, it would be well to restate this conclusion It is not the presence or absence of the Y chromosome in the genotype of an individual that determines whether it will possess incomplete or complete Y-1 Rather, the presence or absence of the Y in the oocyte of a female determines whether her offspring will have incomplete or complete Y-1 Apparently the presence of a Y chromosome in an oocyte so affects the intranuclear or cytoplasmic, nonchromosomal constituents of the oocyte that the individual ultimately developing from the ovum produced by that oocyte possesses an incomplete Y-1, regardless of whether he (or she) himself possesses a Y chromosome in his genotype If a Y chromosome is not present in an oocyte, the progeny produced possesses complete Y-1

Since the antigenic specificity of complete and incomplete Y-1 is the same, the difference would appear to reside in the gross physical attributes of the molecule to which the specificity is attached The Y chromosome is heterochromatin, and Schultz[42] has demonstrated that the presence of a Y chromosome in an oocyte causes the production of ribonucleic acid (RNA) of different base constitution than is produced in its absence It is tempting to suggest that heterochromatin in general is concerned with the production of RNA, which in turn influences the grosser aspects of protein synthesis In terms of the two-stage hypothesis of protein synthesis outlined above, this would involve events during the first stage

The specificity of Y-1, on the other hand, is most probably a reflection of smaller chemical groupings not affected by the grosser physical differences between the complete and incomplete forms (see Pressman[43]) While the present analysis tells us only that there are no genotypic differences between Oregon R and YA in this respect, it is probable that the specificity of Y-1 is the product of one or more euchromatic loci on the X chromosome or autosomes Perhaps euchromatic loci, which in general are noted for their specific effects, are concerned with the production of a different type of RNA, controlling those aspects of protein synthesis involving the determination of the chemical groupings responsible for antigenic or enzymatic specificity In terms of our two-stage hypothesis, such events would probably occur during the second stage

In any event, the production of Y-1 involves genic interactions of a most complex sort, in many ways reminiscent of the interactions observed in the

precipitate is ever observed with antigens of YA males or females. In spite of this, the antigenic extracts of all four genotypes inhibit formation of the precipitate when the Bjorklund technique is applied. From these observations it is concluded that the Y-1 specificity is represented in the Oregon R stock by an antigen capable of inducing antibody formation and capable of uniting to form visible precipitate, that is, a complete antigen (TABLE 8). In the YA stock, on the other hand, the Y-1 specificity is represented by an antigen of different immunological properties: it is capable of inducing antibody formation and will unite with antibody to produce inhibition without the formation of precipitate. To describe these properties we use the term "incomplete" antigen (TABLE 8).

The physical basis for the differences in the immunological properties of complete and incomplete Y-1 is not yet clear. Both forms of the antigen are nondialyzable, but there may be a difference in their solubility in 0.85 per cent NaCl. A physical difference is evident from the observation that the complete form resists dialysis against water and lyophilization, while the incomplete form is frequently inactivated by these procedures. It should be stressed, however, that the specificity of the two forms is the same.

The specificity is obviously associated with neither the number of X chromosomes, nor with the presence or absence of the Y chromosome. It must be attributed to some part of the genotype that is the same in both stocks. We shall later suggest what this is. The difference in the form of the antigen, complete or incomplete, likewise cannot be attributed to either the number of X chromosomes or the presence or absence of Y chromosome. It is a difference that is characteristic of the stocks, but not of the individual genotypes comprising those stocks (X/X and X/Y in Oregon R, $\overline{XX}$/Y and X/Y in YA).

The difference between the two stocks is maternal. Oregon R females are the mothers of Oregon R flies and YA females are the mothers of YA flies. The two types of females differ in two respects: (1) their cytoplasms, and consequently the cytoplasms transmitted to their offspring, are different, and (2) they differ with respect to the presence or absence of the Y chromosome in their genotype, which could also affect their progeny. The stock difference in the form of Y-1 could therefore be attributed to either of two causes: (1) it could represent a case of cytoplasmic inheritance, that is, involving a self-perpetuating cytoplasmic system, or (2) it could result from an effect of the Y chromosome in the oocyte of the mother on her offspring.

To distinguish between these two possibilities, we have derived a new stock in which the females possess YA cytoplasm but lack a Y chromosome (TABLE 9). This was done by making use of an attached X-Y chromosome (X·Y),[41] inserted into the isogenic Oregon R background. By mating YA females ($\overline{XX}$/Y) with X·Y males $\overline{XX}$/O females were obtained, possessing YA cytoplasm (transmitted by their mothers) but lacking a Y. Matings of these with X·Y males produce $\overline{XX}$/O females and X·Y/O males, which serve as a source of antigenic extract. The Y-1 antigen is still present, but in complete form. If its form was the result of cytoplasmic inheritance we should expect it to be incomplete, since the cytoplasm of these flies is YA in origin. It

TABLE 10

CONVENTIONAL HYPOTHESIS NONALLELES

| Inbred Strain A | Inbred Strain B |
|---|---|
| AABBcc | aabbCC |
| Antigens 1 and 2 | Antigen 3 |

$F_1$

AaBbCc
Antigens 1, 2, and 3

| $F_2$ | | A BC | |
|---|---|---|---|
| 27/64 A-B-C- | Antigens 1, 2, and 3 | 1/2 A-B-Cc | Antigens 1, 2, and 3 |
| 9/64 A-B-cc | Antigens 1 and 2 | 1/2 A-B-cc | Antigens 1 and 2 |
| 9/64 A-bbC- | Antigens 1 and 3 | B BC | |
| 9/64 aaB-C- | Antigens 2 and 3 | | |
| 3/64 A-bbcc | Antigen 1 | 1/4 AaBbC- | Antigens 1, 2, and 3 |
| 3/64 aaB-cc | Antigen 2 | 1/4 AabbC- | Antigens 1 and 3 |
| 3/64 aabbC- | Antigen 3 | 1/4 aaBbC- | Antigens 2 and 3 |
| 1/64 aabbcc | None | 1/4 aabbC- | Antigen 3 |

TABLE 11

INTERACTION HYPOTHESIS NONALLELES

| Inbred Strain A | Inbred Strain B |
|---|---|
| AABBcc | aabbCC |
| Antigen 1 | Antigen 2 |

$F_1$

AaBbCc
Antigens 1 and 2

| $F_2$ | | A BC | |
|---|---|---|---|
| 27/64 A-B-C- | Antigens 1 and 2 | 1/2 A-B-Cc | Antigens 1 and 2 |
| 9/64 A-B-cc | Antigen 1 | 1/2 A-B-cc | Antigen 1 |
| 9/64 A-bbC- | Antigen 2 | | |
| 9/64 aaB-C- | Antigen 2 | B BC | |
| 3/64 aabbC- | Antigen 2 | 1/4 AaBbC- | Antigens 1 and 2 |
| 3/64 A-bbcc | Neither | 1/4 AabbC- | Antigen 2 |
| 3/64 aaB-cc | Neither | 1/4 aaBbC- | Antigen 2 |
| 1/64 aabbcc | Neither | 1/4 aabbC- | Antigen 2 |

antigen (antigen 1), and that *C* produces an antigen (antigen 2) without interaction with either of the two other loci The distribution of genotypes in succeeding generations would be the same as in the preceeding case, but only two instead of three antigens would be involved (TABLE 11)

If, now, we consider the transplantation results that would be expected on the basis of each of the hypotheses, a striking observation emerges (TABLE 12) In *both* cases, isotransplants would be accepted (+), homotransplants between the inbred lines would be rejected (−), transplants of tissue from either parental strain would be rejected by $F_1$ animals, transplants of tissue from either parental strain into $F_2$ or backcross animals would be accepted by the same fraction of hosts, and tissue from the $F_1$ would be accepted by all $F_1$ animals, rejected by either parental strain, and accepted by the same fraction of $F_2$ and BC animals In both cases, tissue from an animal of the $F_2$ or later hybrid generations would be accepted by all $F_1$ animals In other words, Snell's "laws of transplantation" would be obeyed in both cases, and the results would not serve to distinguish between the two alternative hypotheses

case of the vermilion pseudoalleles These cases serve to illustrate the complexities involved in gene-antigen relationships

*The Methodology of Transplantation Experiments*

We may now return to the original question Why are gene-antigen relationships so complex in *Drosophila*, but apparently so simple in transplantation immunogenetics? Either there is a difference in principle between the genetic control of antigens in *Drosophila* and the genetic control of the antigens responsible for transplantation specificity, or the difference is only apparent and is attributable to a difference in methodology Incidentally, the same problem arises in connection with the immunogenetics of erythrocytic antigens[17, 28] As I shall demonstrate elsewhere, the answer is in principle apparently the same in both cases

It is possible that there is a real difference in the genetic control of transplantation antigens on the one hand and of our *Drosophila* antigens on the other Billingham *et al*[9] have suggested that the transplantation antigens are desoxyribonucleoproteins (DNA proteins) of nuclear origin, while our antigens are most probably of cytoplasmic origin If transplantation antigens are DNA proteins, they are either the genes themselves or primary gene products, and it would be expected that their production would not involve genic interaction However, Makinodan *et al* report, elsewhere in this monograph, that the antigens involved in bone-marrow transplantation are of diverse nature, some being associated with the microsomal fraction Furthermore, while not decisive, the concept of the unity of biological phenomena would make it very surprising if such a fundamental difference existed in gene-antigen relationships in the two cases We are led, therefore, to a critical examination of the methodology of transplantation experiments

In a typical transplantation experiment, reciprocal transplantations are made between members of two inbred strains and their $F_1$, $F_2$, and backcross progeny The results are scored in terms of the acceptance or rejection by the host of the transplanted tissue, and have been summarized by Snell[44] in the form of six "laws" The point that I wish to demonstrate is that these laws are consistent both with the conventional hypothesis of dominant histocompatibility genes producing the effects without interaction, and with the hypothesis of genic interaction To do so, we shall consider two specific cases, one dealing with nonalleles and one dealing with alleles

In the first case, let us suppose that we are dealing with two inbred strains differing with respect to three nonlinked loci determining the production of tissue antigens, such that strain A is *AABBcc* in genotype and strain B is *aabbCC* According to the conventional hypothesis *A*, *B*, and *C* should each produce an antigen without interaction (antigens 1, 2, and 3 respectively) The distribution of genotypes and antigens in succeeding generations that would be expected on the basis of the conventional hypothesis is given in TABLE 10

A different situation would obtain, however, if genic interaction prevailed On the basis of our experience with *Drosophila* it would be reasonable to consider the possibility, for example, that *A* and *B* interact to produce a single

TABLE 10

CONVENTIONAL HYPOTHESIS: NONALLELES

| Inbred Strain A | Inbred Strain B |
|---|---|
| AABBcc | aabbCC |
| Antigens 1 and 2 | Antigen 3 |

$F_1$

AaBbCc
Antigens 1, 2, and 3

| $F_2$ | | A BC | |
|---|---|---|---|
| 27/64 A-B-C- | Antigens 1, 2, and 3 | 1/2 A-B-Cc | Antigens 1, 2, and 3 |
| 9/64 A-B-cc | Antigens 1 and 2 | 1/2 A-B-cc | Antigens 1 and 2 |
| 9/64 A-bbC- | Antigens 1 and 3 | B BC | |
| 9/64 aaB-C- | Antigens 2 and 3 | 1/4 AaBbC- | Antigens 1, 2, and 3 |
| 3/64 A-bbcc | Antigen 1 | 1/4 AabbC- | Antigens 1 and 3 |
| 3/64 aaB-cc | Antigen 2 | 1/4 aaBbC- | Antigens 2 and 3 |
| 3/64 aabbC- | Antigen 3 | 1/4 aabbC- | Antigen 3 |
| 1/64 aabbcc | None | | |

TABLE 11

INTERACTION HYPOTHESIS: NONALLELES

| Inbred Strain A | Inbred Strain B |
|---|---|
| AABBcc | aabbCC |
| Antigen 1 | Antigen 2 |

$F_1$

AaBbCc
Antigens 1 and 2

| $F_2$ | | A BC | |
|---|---|---|---|
| 27/64 A-B-C- | Antigens 1 and 2 | 1/2 A-B-Cc | Antigens 1 and 2 |
| 9/64 A-B-cc | Antigen 1 | 1/2 A-B-cc | Antigen 1 |
| 9/64 A-bbC- | Antigen 2 | | |
| 9/64 aaB-C- | Antigen 2 | B BC | |
| 3/64 aabbC- | Antigen 2 | 1/4 AaBbC- | Antigens 1 and 2 |
| 3/64 A-bbcc | Neither | 1/4 AabbC- | Antigen 2 |
| 3/64 aaB-cc | Neither | 1/4 aaBbC- | Antigen 2 |
| 1/64 aabbcc | Neither | 1/4 aabbC- | Antigen 2 |

antigen (antigen 1), and that *C* produces an antigen (antigen 2) without interaction with either of the two other loci. The distribution of genotypes in succeeding generations would be the same as in the preceeding case, but only two instead of three antigens would be involved (TABLE 11).

If, now, we consider the transplantation results that would be expected on the basis of each of the hypotheses, a striking observation emerges (TABLE 12). In *both* cases, isotransplants would be accepted (+), homotransplants between the inbred lines would be rejected (−), transplants of tissue from either parental strain would be rejected by $F_1$ animals, transplants of tissue from either parental strain into $F_2$ or backcross animals would be accepted by the same fraction of hosts, and tissue from the $F_1$ would be accepted by all $F_1$ animals, rejected by either parental strain, and accepted by the same fraction of $F_2$ and BC animals. In both cases, tissue from an animal of the $F_2$ or later hybrid generations would be accepted by all $F_1$ animals. In other words, Snell's "laws of transplantation" would be obeyed in both cases, and the results would not serve to distinguish between the two alternative hypotheses.

Table 12

Predicted Transplantation Results Nonalleles

| Host | Transplanted tissue | | |
|---|---|---|---|
| | A | B | $F_1$ |
| A<br>B<br>$F_1$ | +<br>−<br>+ | −<br>+<br>+ | −<br>−<br>+ |
| $F_2$ | 9/16 +<br>7/16 − | 3/4 +<br>1/4 − | 27/64 +<br>37/64 − |
| A BC | + | 1/2 +<br>1/2 − | 1/2 +<br>1/2 − |
| B BC | 3/4 −<br>1/4 + | + | 3/4 −<br>1/4 + |

Table 13

Conventional Hypothesis Alleles

| Isogenic strain | IR strain |
|---|---|
| $H^aH^a$ | $H^bH^b$ |
| Antigen 1 | Antigen 2 |

$F_1$

$H^aH^b$

Antigens 1 and 2

| A BC | $F_2$ | B BC |
|---|---|---|
| 1/2 $H^aH^a$ Antigen 1<br>1/2 $H^aH^b$ Antigens 1 and 2 | 1/4 $H^aH^a$ Antigen 1<br>2/4 $H^aH^b$ Antigens 1 and 2<br>1/4 $H^bH^b$ Antigen 2 | 1/2 $H^bH^b$ Antigen 2<br>1/2 $H^aH^b$ Antigens 1 and 2 |

When we come to consider the effects of alleles, a typical case would be that of an isogenic strain of the genotype $H^aH^a$ and an IR strain of genotype $H^bH^b$ In accordance with the situation obtaining in the case of H-2 alleles in the mouse,[45] the conventional view of gene-antigen relationships would propose that each allele produces its antigenic effect without interaction with the other (TABLE 13) On the basis of our experience with *Drosophila*, and on the basis of a case of interallelic interaction in pigeons,[46] it might be proposed as an alternative that the two alleles interact in the heterozygote to produce an interaction antigen (antigen 3) as well as the antigens that they each produce in the respective homozygotes (antigens 1 and 2) TABLE 14 summarizes this hypothesis As in the previous case, the predicted distribution of genotypes in succeeding generations is the same for both hypotheses, but the distribution of antigens is different

Regardless of this fact, the predicted results of transplantation experiments is the same in both cases (TABLE 15) Such results, therefore, would once again fail to distinguish between the two hypotheses The situation

TABLE 14

INTERACTION HYPOTHESIS ALLELES

| Isogenic strain | IR strain |
|---|---|
| $H^aH^a$ | $H^bH^b$ |
| Antigen 1 | Antigen 2 |

$F_1$

$H^aH^b$

Antigens 1, 2, and 3

| A BC | $F_2$ | B BC |
|---|---|---|
| 1/2 $H^aH^a$ Antigen 1 | 1/4 $H^aH^a$ Antigen 1 | 1/2 $H^bH^b$ Antigen 2 |
| 1/2 $H^aH^b$ Antigens 1, 2, and 3 | 2/4 $H^aH^b$ Antigens 1, 2, and 3 | 1/2 $H^aH^b$ Antigens 1,2, and 3 |
| | 1/4 $H^bH^b$ Antigen 2 | |

TABLE 15

PREDICTED TRANSPLANTATION RESULTS ALLELES

| Host | Transplanted tissue | | |
|---|---|---|---|
| | Isogenic | IR | $F_1$ |
| Isogenic | + | − | − |
| IR | − | + | − |
| $F_1$ | + | + | + |
| $F_2$ | 3/4 +<br>1/4 − | 3/4 +<br>1/4 − | 1/2 +<br>1/2 − |
| A BC | + | 1/2 +<br>1/2 − | 1/2 +<br>1/2 − |
| B BC | 1/2 +<br>1/2 − | + | 1/2 +<br>1/2 − |

would not be altered if, as in the case of the H-2 alleles,[34] each allele produced more than one antigenic effect

While the two cases we have just examined are specific, they serve the purpose of illustrating the more general conclusion that the classic methods of transplantation immunogenetics are incapable of excluding the hypothesis of genic interaction in antigen production This incapability is attributable largely to the fact that the results are restricted to a binary system of observations, acceptance or rejection Such observations yield information that may be used to calculate the number of histocompatibility loci with respect to which two inbred lines differ, but they cannot reveal the number of antigens involved nor the mode of their genetic determination

More recently Snell has developed a series of new methods designed to identify particular histocompatibility loci and to disclose allelic differences at those loci[7, 12, 47, 48] These methods have brilliantly fulfilled their purpose, but the very fact that they were designed for the study of the antigenic effects of particular loci prevents them from disclosing genic interaction The use of IR (coisogenic) strains, for example, facilitates the elucidation of the antigenic

effects of genic substitution at a single locus, but since the residual genotype is the same in an isogenic strain and its IR derivative, no information is obtained regarding the possibility of interaction between the locus in question and residual loci Similarly, the "linkage test," the "$F_1$ test," and the "immunization test" are admirably suited for the identification of allelic differences at particular histocompatibility loci, but they do not yield information regarding the possible interactions of these alleles with genes at other loci or with each other These comments are not meant as criticisms of these beautiful methods, for they accomplish their aims in an admirable fashion, but simply as an evaluation of their limitations

In recent years, transplantation methodology has also been supplemented by direct antigenic analysis, made possible by the development of methods using red-cell or white-cell agglutinins [49, 50] These methods are potentially capable of detecting interaction antigens Their use, however, has so far been largely restricted to antisera obtained by the inoculation of tissue indigenous to an isogenic strain into its IR counterpart and vice versa [34] The limitations of the IR method are therefore imposed on the results that may be obtained

*Discussion*

From the considerations of the preceeding section it may be concluded that the failure of transplantation experiments to demonstrate genic interaction in antigen production is probably methodological in source This conclusion, however, does not establish the occurrence of genic interaction It therefore becomes important to consider instances which may involve such a phenomenon, and to propose methods capable of demonstrating it

One of the consequences of the hypothesis of genic interaction is that a given histocompatability gene should produce different antigenic effects on different genetic backgrounds, provided that these backgounds differ with respect to the residual loci with which the histocompatability gene in question interacts It might therefore be proposed that studies be performed in which histocompatability genes are transferred from their original genetic backgrounds to a series of different ones by crosses between unrelated strains

In a sense, some such studies have already been performed It may be that some of the difficulties encountered with the "weak" histocompatability genes (H-1 and H-3 in the mouse) are attributable to genic interaction In these cases, grafts sometimes survive even when donor and host possess different alleles [7, 45, 51] Some of these unexpected acceptances may be attributable to changed antigenicity due to interaction of the histocompatability gene with other genes, although other factors are certainly also involved

The most extensive evidence of this sort, however, comes from studies of the H-2 locus and, at first sight, this is not favorable for the hypothesis of gene interaction Snell lists the H-2 alleles present in 19 different isogenic strains of the mouse [7] In some cases the same H-2 allele is present in several (as many as 6) different strains, and apparently produces the same antigens in all Closer examination reveals, however, that these strains are frequently related,

and this may be the case not only with respect to the H-2 locus, but also with respect to residual loci with which it might interact In other words, it is quite possible that the residual genotypes of the strains that have been examined have not differed with respect to pertinent loci The probability that this might occur would be large if only a few interacting loci are involved Beyond this, the H-2 locus may be similar in its antigenic effects to the vermilion loci in *Drosophila* The latter, it will be recalled, produce antigenic effects apparently without interaction, but also participate in the production of interaction antigens Indeed, the peculiar "strength" of the H-2 locus suggests that it may be atypical, rather than representative of the majority of histocompatability genes

A phenomenon that almost certainly involves genic interaction, particularly in view of our studies of the effects of X-chromosome dosage in *Drosophila*, is that of antigenic simplification of tumors [8] Hauschka *et al* have demonstrated that such changes in the specificity of tumors are accompanied by aneuploid increases in chromosome number, that is, by changes in chromosome balance [52-55] Translocations (that is, exchanges between chromosomes) are apparently frequent [56] In view of these findings, deletion of chromosome segments involved in antigen determination, position effects on antigen differentiation, or impairment of antigen synthesis by over-all chromosome imbalance have been suggested as possible mechanisms of antigenic simplification The last of these suggestions is documented by the antigenic effects of changes of X-chromosome dosage in *Drosophila* The possibility that position effects resulting from chromosome rearrangement might be involved also could mean disturbances of genic interactions Information concerning position effects on antigens in *Drosophila* should be forthcoming from a series of studies in which we are currently engaged

It is of particular interest to note that aneuploid increases in chromosome number have been demonstrated to be accompanied by the disappearance of some, but not all, of the antigens governed by the H-2 locus [57, 58] If the suggestion made above regarding the manner in which the H-2 locus produces its effects were correct, such a partial loss could readily occur if the effects of interacting loci were changed In any event, it would seem very much worthwhile to pursue studies of antigenic simplification as a source of information regarding genic interaction in antigen production

Another phenomenon that merits further attention was first described by Barrett and Deringer [59-61] This is a stable change either in the direction of decreased or increased specificity displayed by certain tumors after a single passage through an $F_1$ hybrid produced by crossing the indigenous strain with a resistant strain [62, 63] The change is due to a host-induced adaptation in individual tumor cells, the inducing agents being humoral factors of the $F_1$ host [64] The possibility that the inducing agents are antibodies should be considered, even though attempts to induce mutations in other organisms by means of antibodies have failed [65-68] On the other hand, the whole phenomenon is reminiscent of bacterial transformation or transduction and may involve the transfer of genetic specificity by means of desoxyribonucleic acid (DNA) from

$F_1$ host to tumor cells. Since the tumor cells can acquire antigenic specificities present in neither of the two parents of the $F_1$, it would also have to be supposed that the introduced DNA interacts with genes already present in the transformed cells to produce new antigenic products.

The most direct way of demonstrating genic interaction in the determination of tissue specificity would be to investigate the antigens of $F_1$ tissues. This could be done either with tissue extracts, using techniques similar to those we have applied to *Drosophila*, or with cells, using the techniques of Gorer and Mikulska[49] or of Amos.[50] It would be necessary, however, to immunize unrelated animals, possibly rabbits, and to subject the resulting antisera to absorption. If an antiserum absorbed with the antigens of both parental strains still was capable of reacting with $F_1$ antigens, evidence of genic interaction would be obtained.

Haldane, who has also considered the possibility of genic interaction in antigen production, has suggested another approach.[69] Making use of the phenomenon of acquired tolerance,[70] he suggests that animals of one inbred strain of mice be pretreated to induce tolerance to the antigens of a second inbred strain. $F_1$ tissue, from animals produced by crossing the two strains, would then be transplanted on the pretreated animals. The rejection of such grafts would be evidence of the presence of interaction antigens in the $F_1$.

Still another approach would be to take advantage of the so-called "runt disease" described in this monograph by Billingham. When new-born mice of the A strain are inoculated with C57 spleen cells they frequently develop the symptoms of this syndrome, presumably because of the response of the introduced spleen cells to host antigens. C57 animals could be rendered tolerant to A antigens by pretreatment. If spleen cells from such tolerant animals induced runt disease in the $F_1$ hybrids produced by crossing C57 with A, evidence would be obtained for the presence of interaction antigens in the $F_1$.*

I urge interested investigators to explore some of these possibilites. The questions involved are sufficiently important to merit attention.

*Summary*

On the basis of transplantation experiments, tissue specificities have been attributed in the past to the production by single genes of their dominant effects without interaction, a process that would result in a simple relationship between gene and specificity. The validity of this view is examined in the light of results of investigations of the genetic control of the specificity of tissue antigens in *Drosphilia melanogaster*. Cases involving interaction of nonalleles (ruby and vermilion), pseudoalleles (vermilion mutants), and alleles (lozenge) are reviewed. New evidence pertaining to qualitative effects on antigenic specificity of X chromosomal dosage and to the role of the Y chromosome (heterochromatin) and cytoplasm is presented. Possible mechanisms of genic interaction in protein synthesis, and the implications of these findings for the genetics of tissue specificity are discussed. Reasons for the failure of trans-

* The author is indebted to Seymour Abrahamson for important elements of this suggestion.

plantation experiments to disclose more complex relationships between gene and specificity are suggested, and possible instances of such relationships are cited Methods capable of demonstrating genic interaction in the determination of transplantation antigens are presented, and interested investigators are urged to perform such studies

## *References*


1 WRIGHT, S 1941 Physiol Revs **21** 487
2 HALDANE, J B S 1933 Nature **132** 265
3 IRWIN, M R 1947 Advances in Genetics **1** 133
4 IRWIN, M R 1955 *In* Biological Specificity and Growth . 55 E G Butler, Ed Princeton Univ Press Princeton, N J
5 WRIGHT, S 1945 Ann Rev Physiol **7** 75
6 LITTLE, C C 1941 *In* Biology of the Laboratory Mouse 279 G D Snell, Ed Blakiston Philadelphia, Pa
7 SNELL, G D 1953 J Natl Cancer Inst **14** 691
8 GORER, P A. 1948 Brit J Cancer **2** 103
9 BILLINGHAM, R E, L BRENT & P B MEDAWAR 1956 Nature **178** 514
10 LITTLE, C C 1951 *In* Genetics in the 20th Century 431 L C Dunn, Ed Macmillan New York, N Y
11 HAUSCHKA, T S 1952 Cancer Research **12** 615
12 SNELL, G D 1948 J Genet **49** 87
13 HALDANE, J B S 1936 J Genet **32** 375
14 WRIGHT, S 1933 Proc Natl Acad Sci **19** 420
15 SNELL, G D 1956 Transplantation Bull **3**. 29
16 MULLER, H J 1936 Drosophila Information Service **6** 7
17 FOX, A S 1949 Genetics **34** 647
18 STADLER, L J 1954 Science **120** 811
19 BENZER, S 1957 *In* The Chemical Basis of Heredity . 70 W D McElroy & B Glass, Eds Johns Hopkins Press Baltimore, Md
20 FISHER, R A. 1954 Heredity **8** 187
21 CHOVNICK, A & A S FOX 1953 Proc Natl Acad Sci **39** 1035
22 BARISH, N & A S FOX 1956 Genetics **41** 45
23 FOX, A S & S B YOON 1958 Transplantation Bull In press
24 FOX, A S, K E FUSCALDO & C G MEAD 1958 In preparation
25 FOX, A. S 1949 J Immunol **62** 13
26 OUDIN, J 1952 Methods in Med Research. **5** 335
27 FOX, A S, S B YOON & C G MEAD 1958 In preparation
28 FOX, A S & T B WHITE 1953 Genetics **38** 152
29 FINKELSTEIN, P & A S FOX 1953 Genetics **38** 166
30 FOX, A S 1954 Nature **173** 350
31 FOX, A S & J B BURNETT 1958 *In* Pigment Cell Biology M Gordon, Ed Academic Press New York, N Y In press
32 HAUROWITZ, F 1950 Chemistry and Biology of Proteins Academic Press New York, N Y
33 GREEN, M M 1954 Proc Natl Acad Sci **40** 92
34 HOECKER, G, S COUNCE & P SMITH 1954 Proc Natl Acad Sci **40** 1040
35 CHOVNICK, A, R J LEFKOWITZ & A S FOX 1956 Genetics **41** 589
36 GREEN, M M & K C GREEN 1949 Proc Natl Acad Sci **35** 586
37 GREEN, M M 1956 Z Abstamm u Vererbungslehre **87** 708
38 FOX, A S 1956 Transplantation Bull **3** 131
39 EICHWALD, E J & C R SILMSER 1955 Transplantation Bull **2** 57
40 HAUSCHKA, T S 1955 Transplantation Bull **2** 154
41 LINDSLEY, D L & E NOVITSKI 1950 Drosophila Information Service **24** 84
42 SCHULTZ, J 1956 Cold Spring Harbor Symposia Quant Biol **21**: 307
43 PRESSMAN D 1957 *In* Molecular Structure and Biological Specificity I L Pauling & H A Itano, Eds American Institute of Biological Sciences Washington, D C
44 SNELL, G D 1953 *In* The Physiopathology of Cancer . 338 F Homburger & W H Fishman, Eds Harper & Bros New York, N Y
45 SNELL, G D & G F HIGGINS 1951 Genetics **36** 306
46 BRYAN, C R & W J MILLER 1953 Proc Natl Acad Sci **39** 412

47 Snell, G D, P Smith & F Gabrielson 1953 J Natl. Cancer Inst 14 457
48 Snell, G D, N Wheeler & M Aaron 1957 Transplantation Bull 4 18
49 Gorer, P A & Z B Mikulska 1954 Cancer Research 14 651
50 Amos, D B 1953 Brit J Exptl Pathol 34 464
51 Counce, S, P Smith, R. Barth & G D Snell 1956 Ann Surg 144 198
52 Hauschka, T S & A Levan 1953 Exptl Cell Research 4 457
53 Hauschka, T S & J Schultz 1954 Transplantation Bull 1 203
54 Hauschka, T S, B J Kvedar, S T Grinnell & D B Amos 1956 Ann N Y Acad Sci 63(5) 683
55 Hauschka, T S 1953 J Natl Cancer Inst 14 723
56 Levan, A 1956 Ann N Y Acad Sci 63(5) 774
57 Hoecker, G & T S Hauschka 1956 Transplantation Bull 3 134
58 Amos, D B 1956 Ann N Y Acad Sci 63(5) 706
59 Barrett, M K & M K Deringer 1950 J Natl. Cancer Inst 11 51
60 Barrett, M K & M K. Deringer 1952 J Natl Cancer Inst 12 1011
61 Barrett, M K, M K Deringer & W H Hansen 1953 J Natl Cancer Inst 14 381
62 Hauschka, T S 1953 J Natl Cancer Inst 15 1530
63 Klein, G, E Klein & L Révész 1954 Transplantation Bull 1 101
64 Klein, E & G Klein 1956 Transplantation Bull 3 136
65 Fox, A S & N K Zeibur 1952 Genetics 37 581
66 Fox, A. S, L H Herskowitz & A. Schalet 1955 Drosophila Information Service 29 116
67 Ryan, F J, P Fried & E Gonsalez 1953 Am Naturalist 87 383
68 Markert, C L & R. D Owen 1954 Genetics 39 818
69 Haldane, J B S 1956 J Genet 54 54
70 Billingham, R E, L Brent & P B Medawar 1956 Phil Trans Roy Soc London B239 357

# INDUCED IMMUNITY TO CANCER CELL HOMOGRAFTS IN MAN*

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## *Introduction*

The term induced immunity is used to designate a heightened capacity of an individual for reaction against a foreign material that follows and is the result of prior exposure to that specific material The term is used in contradistinction to natural immunity or natural resistance, which may be defined as the sum of those defense mechanisms that are available to an individual upon first exposure to a foreign material

It has been previously reported[1, 2] that human volunteer recipients show a marked difference in their natural resistance to subcutaneously homotransplanted cancer cells according to whether they are normal healthy adults or patients with advanced debilitating neoplastic disease In brief, normal recipients responded to implanted human cancer cells with a marked local inflammatory reaction and rapid complete regression of the implants within a maximum period of 3 to 4 weeks This is the response that one would predict for any homologous tissue transplant In striking contrast, however, those recipients that had advanced cancer showed little or no acute inflammatory response, the implanted cancer cells grew progressively for a period of 3 weeks or more before regression started, and some individuals failed to reject implanted cancer cells over periods of observation between 6 weeks and 6 months When regression did occur it was accompanied by a predominantly round-cell infiltrate, without acute inflammatory changes

## *Methods*

All cancer cells were established lines maintained for at least 3 years and as long as 8 years in serial passage in tissue culture or in heterologous hosts (chick embryos or cortisone-treated rats or hamsters) or in various alternating combinations of these Normal cells were grown in tissue culture for only 1, 2, or 3 transplant generations (total periods of 3 days to 1 month) All implants used for the study of induced immunity were prepared from tissue cultures by trypsinizing the cells to remove them from the glass, washing twice in balanced salt solution to remove the trypsin and protein nutrients, counting in a hemocytometer, and adjusting volume to yield the desired cell concentrations The number of cells inoculated at each site was kept constant between 1 and 3 million in a volume of 0 5 ml of Gey's salt solution or Eagle's synthetic medium

Tissue culture was on glass, without plasma or other supporting medium The nutrient medium most commonly used was Eagle's synthetic medium 80

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per cent and human serum 20 per cent, but at various times other media have been substituted involving use of Gey's salt solution, chick embryo extract, human ascitic fluid, and horse serum

The cell types used for the testing of induced immunity were HEp 2 and HEp 3[3] These are both human epidermoid carcinoma cells from primary epidermoid cancers of larynx and buccal mucosa, respectively The following additional cancer cell types were used in primary inoculations HEp 1, HS 1, J 111, Deac 6, and HeLa[1,2]

Some of the primary implants utilized cells propagated in heterologous hosts instead of tissue cultures Individuals receiving such implants have not been presented separately in the present study, because they showed no difference from recipients who were injected with tissue-cultured cells of the same type and because, in a study of their primary responses, it was established that the characteristics of the cell line were not affected by the site of cultivation

Human amnion cells were obtained from placentas from normal deliveries Normal human embryonic fibroblasts were cultivated from human embryos of various ages obtained following therapeutic abortions from sources outside this institute and with detailed medical histories unknown to us

All implants were subcutaneous, on either the flexor surface of the forearm or the anterior surface of the thigh At least 2 and sometimes as many as 4 implants of the same or different cell types were made on an individual simultaneously Previous experience has shown no apparent difference in the growth of transplants related to the number of types of cells inoculated at any one time Whenever repeat implants were being made, simultaneous inoculation using aliquots of the same cell suspension were made in previously uninoculated individuals so that comparison could be made with simultaneous controls in addition to comparison with previous reactions in the same individual Nodule diameters were measured through the skin with an ordinary centimeter ruler Maximum and minimum diameters were recorded to the closest millimeter In FIGURES 1 to 7 the number of implants upon which mean nodule diameters are computed at each time interval is indicated by the adjacent numeral When an individual recipient received 2 implants of a given cell type, one was usually removed for biopsy before day 14, while the other was left until at least day 14 for observation of size Consequently, the number of nodules indicated at 14 days usually indicates the number of individual recipients upon which each curve is based

Biopsies were performed by excision under local procaine anesthesia Histological sections with hematoxalyin-eosin stain were made from all biopsies and, unless the excised nodule was less than 0 5 cm in diameter, one half was minced and planted in tissue culture in an attempt to reisolate the transplanted cell When cells of the transplanted type grew out in culture, their identity was confirmed by transplantation into cortisone-treated rats and by subsequent histological study of the tumors so produced

In the tissue culture tests for presence of cytotoxic antibodies, the test sera were added to nutrient medium to yield final serum dilutions of 1 12 or 1 30 and substituted for the routine medium in established tube cultures of 5 lines

of human cells The living cultures were examined microscopically daily for evidence of cell damage as compared with parallel control cultures

Additional studies of cytotoxic activity against HEp 3 cells were also carried out by Helene Toolan In her technique the sera were mixed with a mince of HEp 3 cells grown in rats to give a final serum dilution of 1 5 This mixture was incubated at 37° C for 1 hour and then inoculated subcutaneously into cortisone-treated rats In this technique cytotoxicity is indicated by retardation or failure of growth of this implant

Standard methods were used for the *in vitro* serologic tests The complement fixation test utilized two 100 per cent hemolysis units of complement, with 4-hour or overnight incubation at 4° C The antigens were tissue-cultured HEp 2 cells after storage in a solid $CO_2$ box and disrupted by a mechanical guide or by ultrasonic irradiation Studies with the Ouchterlony agar-diffusion technique were done by Leonhard Korngold, using aqueous extracts of tissue-cultured HEp 2 and J 111 cells as antigens

*Results*

In studying the phenomenon of induced immunity it has been necessary, because of the marked difference in response to primary implants in the 2 groups of recipients, to study normal volunteer recipients and cancer patient volunteers as separate groups Primary implants are compared with repeat implants (inoculated 2 to 5 months later) on the basis of nodule size and persistence, histology, and recovery of the implanted cells by tissue culture Present evidence of induced immunity rests on these observations Limited serologic studies are also presented

*Nodule Size Data*

*Normal Recipients* FIGURE 1 illustrates nodule size and persistence in the same group of normal volunteers inoculated with the same cell type (HEp 3 or HEp 2 or HEp 1) on 3 different occasions (these nodules consist of inflammatory tissue in addition to the cancer cells if any see histological data below) The nodules that appeared at inoculation sites after primary implantation increased progressively in size for 2 weeks, regressed during the third week, and disappeared completely during the fourth week On repeat implantation the nodules did not differ significantly in size through day 7, but thereafter there was no further increase and usually there was definite regression by day 14

These two characteristics of the nodules that appeared at sites of repeat implantation of the same cell type in normal recipients (that is, failure to increase in size after day 7, and the consequent smaller maximum size) were observed in every study and in every individual so tested Such acceleration of nodule regression seems analogous to the second-set reaction in skin grafting

The repeat implants were usually made on the opposite arm from the primary implant. Thus, the accelerated rejection cannot be attributed to local tissue changes resulting from the first implant, but must be a systemic change The few recipients who took both primary and repeat implants in the same arm also showed the accelerated regression, but the data are insufficient to permit

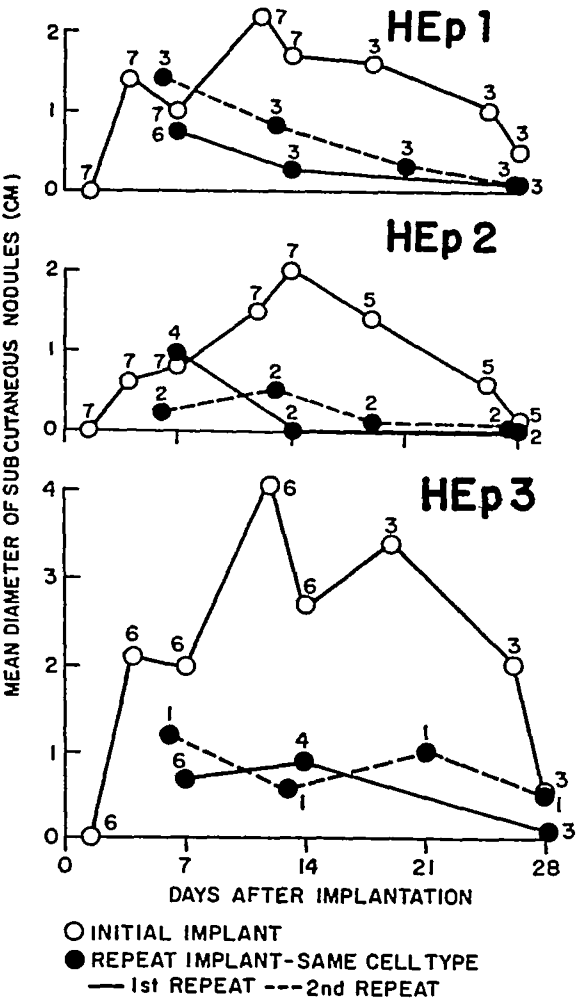



FIGURE 1 Nodule size curves in a group of normal volunteers who received repeat implants of the same cell line used for primary implants (HEp 1, HEp 2, or HEp 3) Note smaller maximum size and rapid regression of repeat implants

quantitative comparison of nodule size in ipsilateral versus contralateral implants

FIGURE 2 illustrates similar results from a study in which previously uninoculated volunteers and others who had received previous implants of the same cell type were inoculated simultaneously, using a single preparation of either HEp 3 or HEp 2 cells Since the same results were obtained using either the same recipients at different times or the identical inoculum in different individuals, it follows that the differences cannot be attributed either to individual variation or to differences in the cell preparation, but must result from a change in host receptivity induced by the previous inoculation of the cells

FIGURE 3 presents combined data from three studies of induced immunity that have been completed to date HEp 1 is omitted from this and subsequent charts because the only study with this cell type is presented in FIGURE 1 In this and in all other presentations of combined data the primary implant data are from individuals who were inoculated at the same time and with the

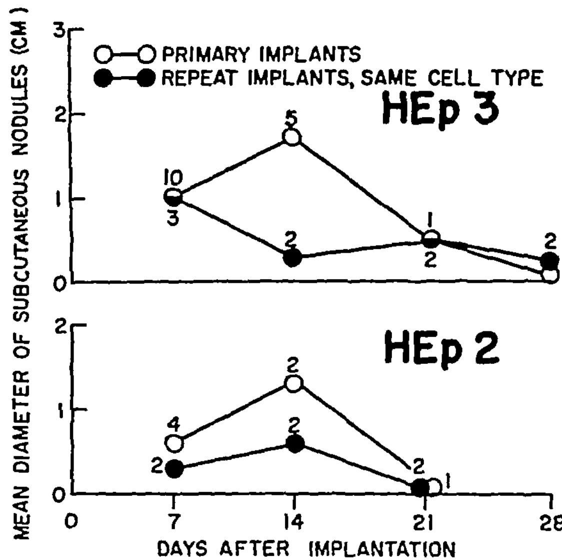


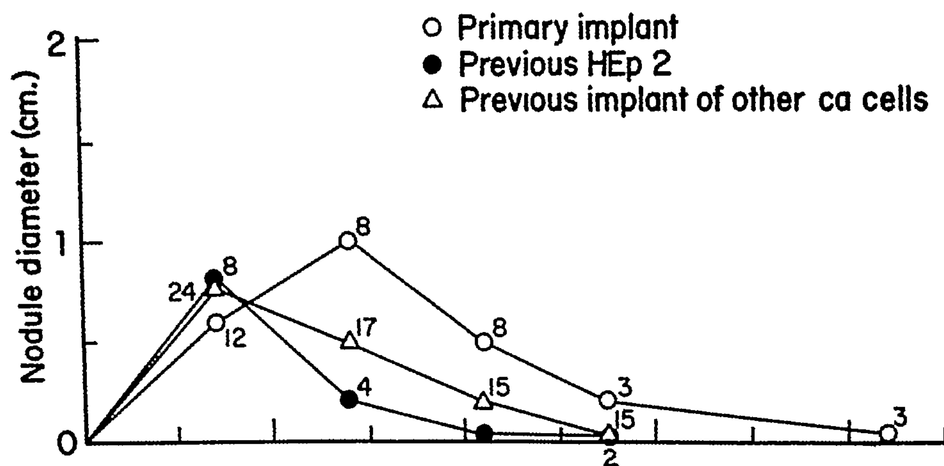

FIGURE 2 Nodule size curves in groups of normal volunteers inoculated simultaneously with identical cell preparations Contrast rapid regression of implants in group previously inoculated with the same cell line (HEp 2 or HEp 3) with the slower evolution of the primary implants

same inoculum as the individuals who received repeat implants The same evidence of accelerated rejection is seen in these combined data as in the individual studies presented in FIGURES 1 and 2.

The third curve in FIGURE 3 illustrates the behavior of implants of the HEp 3 or HEp 2 cells in normal individuals who were never previously exposed to this particular type of cell, but who had received previous implants of other types of cancer cells This group likewise showed accelerated rejection as compared to the primary implant group The other cell types used in the primary implants were HEp 1, HeLa, and HS 1 Separate analysis for each of the cell types used for the primary implants showed that the accelerated rejection occurred with each type, although with both HEp 3 and HEp 2 the rate of rejection of second implants was fastest when the previous implant was of the same cell type

We interpret this accelerated rejection of second implants as an indication of induced immunity If this be correct it follows that the induced immunity is not specific for a single cell type, but shows at least a partial cross-over to the other types of cancer cells that have been studied This suggests that the cancer cells, although not antigenically identical, contain some antigens in common

It becomes of interest then to compare the response of recipients who previously have been injected with only normal human cells (FIGURE 4) The difficulty of obtaining large quantities of normal human cells has restricted the number of such studies, but in the limited experience to date (4 individuals) the behavior of the secondary cancer cell implants has been of the primary type rather than the accelerated rejection that we associate with a second-implant immune response The normal cells were human embryonic fibroblasts or amnion cells in early tissue culture passage It would appear that



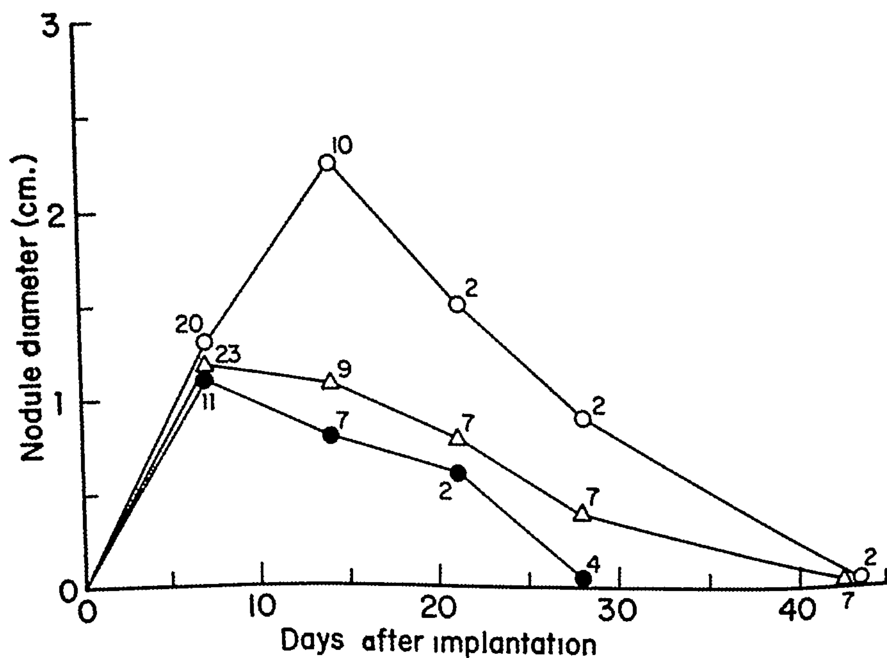




FIGURE 3 Combined nodule size data from all repeat implant studies in normal volunteers and the simultaneous primary implants Note that rejection was most rapid when the primary implants were of the same cell line, but accelerated rejection is also seen following primary implants of other cancer cells

these normal cells do not contain antigens of the type or in the quantity that immunized against subsequent implants of cancer cells

The implantation of HEp 2 or HEp 3 cells that had been killed by alternate freezing and thawing or by incubation at 56° C for 10 min did not elicit an inflammatory reaction or nodule formation, as was also true for the normal cells However, these killed cancer cells did induce an immune-type reaction similar in degree to that induced by the living cell (FIGURE 5)

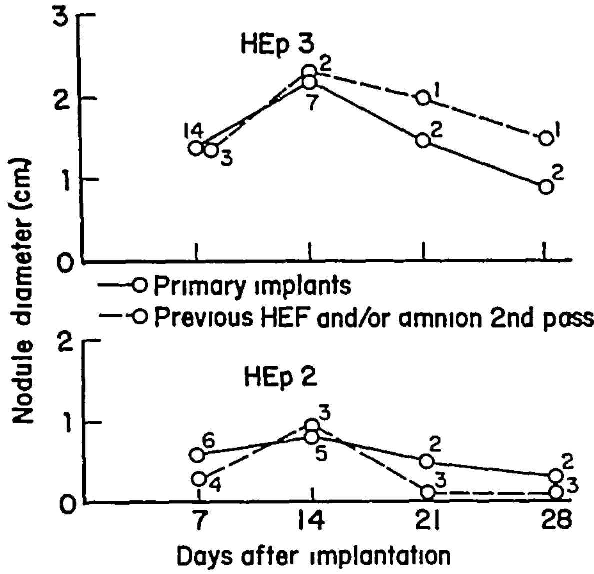



FIGURE 4 Normal volunteers previously implanted with normal human fibroblasts or amnion cells reacted to subsequent cancer cell (HEp 2 or HEp 3) implants in the same way as simultaneous recipients of primary cancer cell implants Compare FIGURE 3, which illustrates accelerated rejection when previous implants were cancer cells

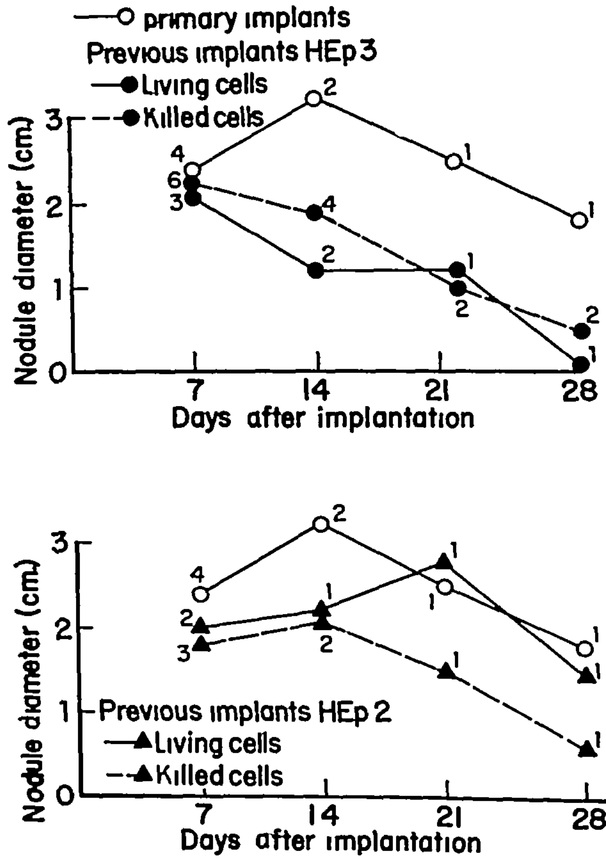



FIGURE 5 Accelerated rejection of cancer cell homotransplants in normal volunteers previously injected with killed cells of same line (HEp 2 or HEp 3)

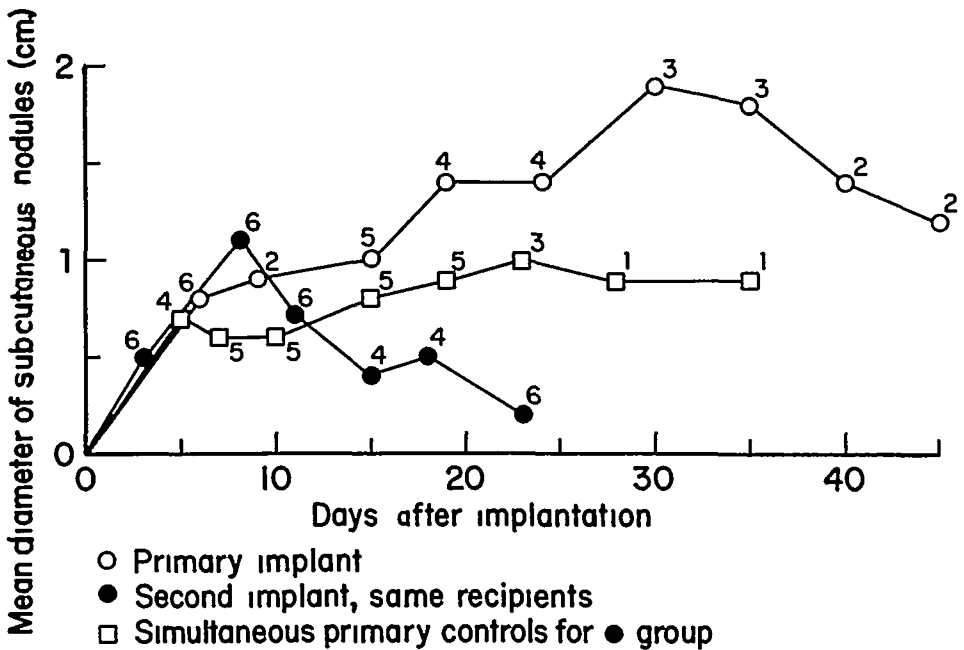



FIGURE 6 Accelerated rejection by cancer patients of repeat implants of cancer cells (HEp 2) as judged by comparison with the primary implants in the same recipients or in simultaneous primary controls Note that the repeat implants were rejected as promptly by these cancer patients as by normal recipients (FIGURES 1, 2, and 3), although rejection of primary implants in cancer patients was very slow

*Cancer patients* Since natural immunity to transplanted cancer cells is so markedly impaired in patients with advanced cancer it was of interest to determine whether, after an implant had eventually been rejected, these patients would show induced immunity Our experience with this group of recipients is small, because only patients with very brief life expectancy were accepted for homotransplantation studies and because of the prolonged period of growth of the primary implants It is further limited to patients whose first implant was HEp 2 cells because those who had a primary inoculation of the more aggressive HEp 3 cell have not, in our experience to date, rejected the implant

FIGURE 6 indicates that repeat implants of HEp 2 cells were rejected promptly by subjects who had previously had HEp 2 implants In this figure there are 2 primary implant control curves the same patients who later received repeat implants, and the simultaneous controls for the repeat implant studies The rapid regression of the repeat implants is in sharp contrast to the longer persistence and growth of the primary implants of the same cell type

FIGURE 7 illustrates the behavior of HEp 3 cell implantations in patients who had previously rejected HEp 2 cell implants Here, too, the implants started to regress in the third week and disappeared completely by 5 weeks, whereas the simultaneous primary controls showed progressive growth well beyond this time

There was no gross inflammatory reaction to the repeat implants in the cancer patients

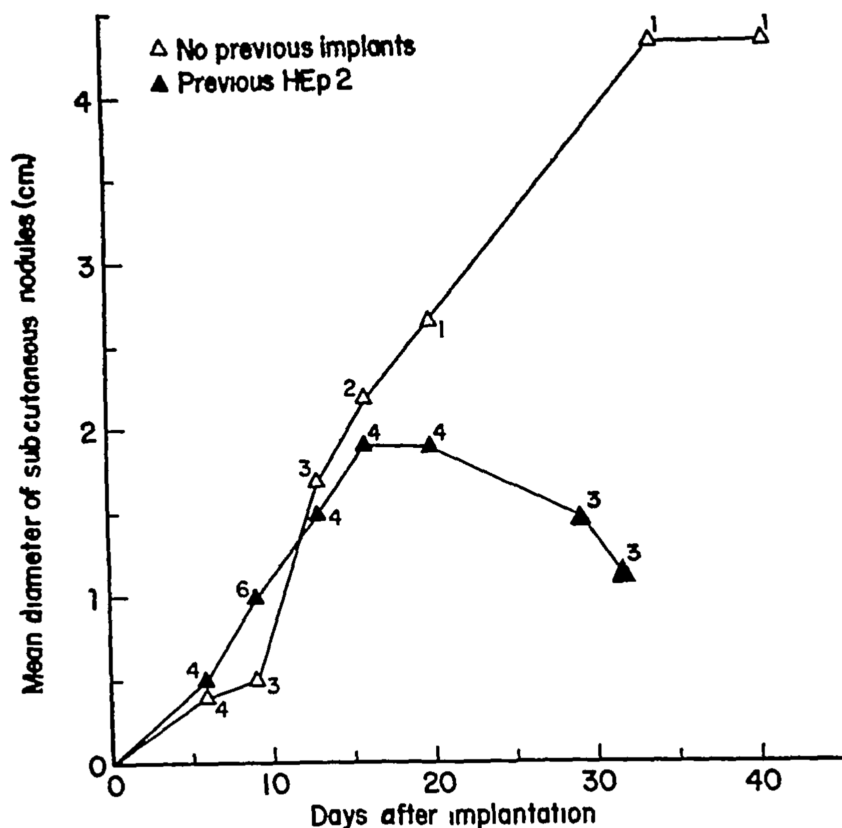


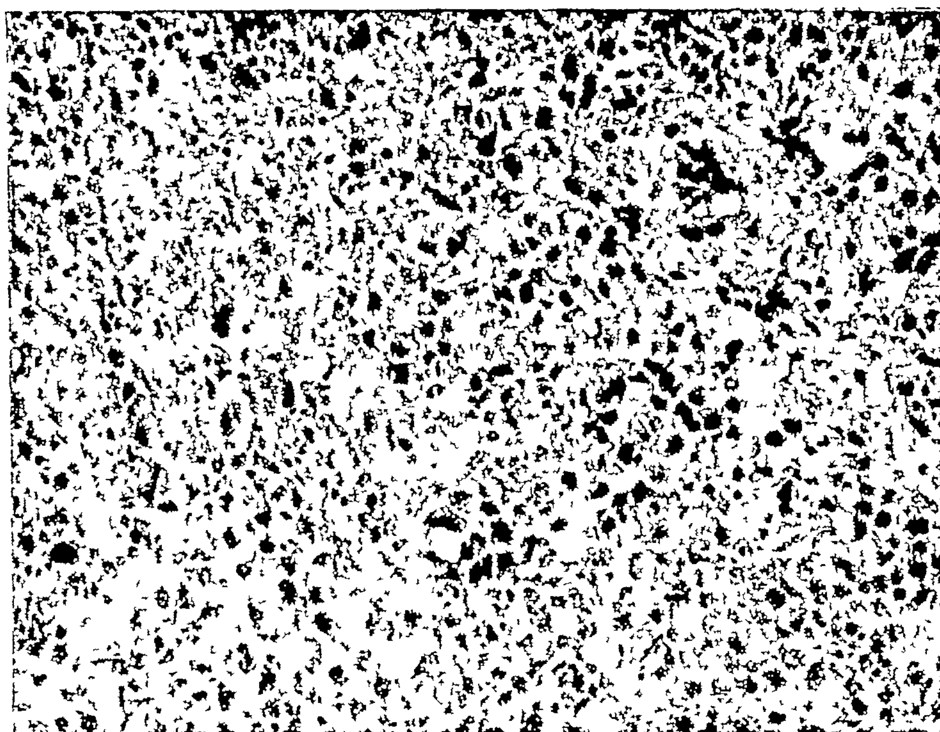

FIGURE 7 Accelerated rejection by cancer patients of implants of HEp 3 cells following primary implants of a different (HEp 2) cancer cell line

*Histological Data*

Since measurements of nodule size are only an indirect reflection of the propagation of the implanted cells, results have also been evaluated on the basis of histological composition of biopsied material The two categories of recipients are considered separately because of the marked difference in their reaction to the implants

*Normal recipients* The nodules that appeared at the sites of cancer cell homotransplants in normal recipients consisted principally of acute inflammatory reaction Mononuclear cell infiltration tended to be more prominent in day-14 biopsies, but even here the acute inflammatory picture was predominant When cancer cells were present they were scattered or in small sheets, but never made up as much as one half of the nodule mass No qualitative difference was apparent in the histological composition of nodules excised from primary or repeat implants However, when biopsies of primary and repeat implants were available from the same individual at the same interval after inoculation, it appeared that in repeat implants cancer cells were frequently absent and in smaller numbers when present FIGURES 8 to 10 illustrate 3 such paired biopsies

Total biopsy data on HEp 3 and HEp 2 implants in normal recipients are indicated graphically in FIGURE 11, using an arbitrary system of grading to permit crude but objective summation of the histological findings A nodule



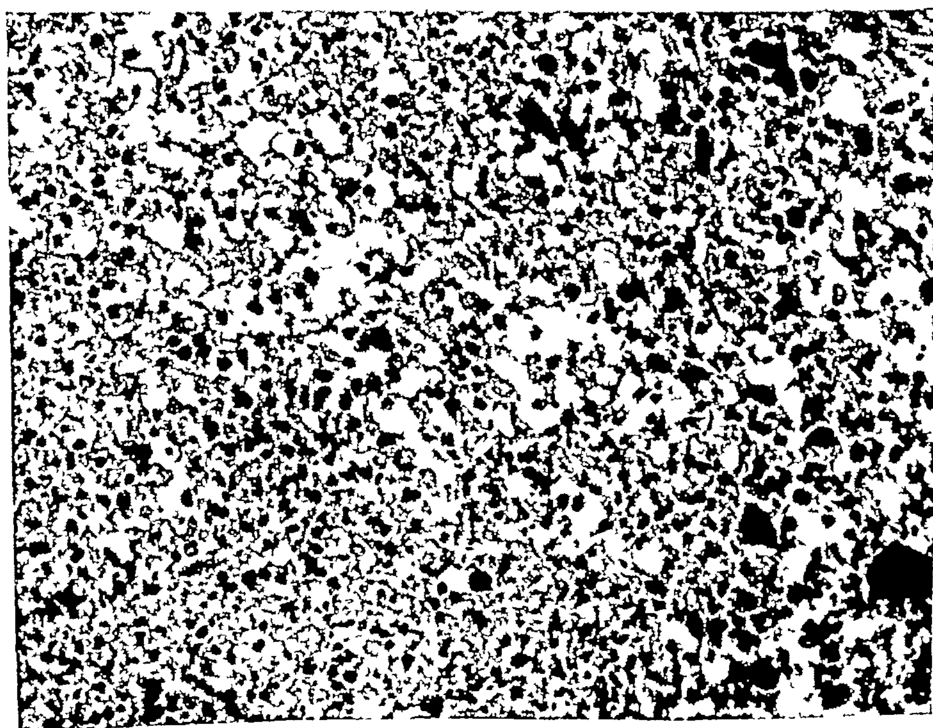
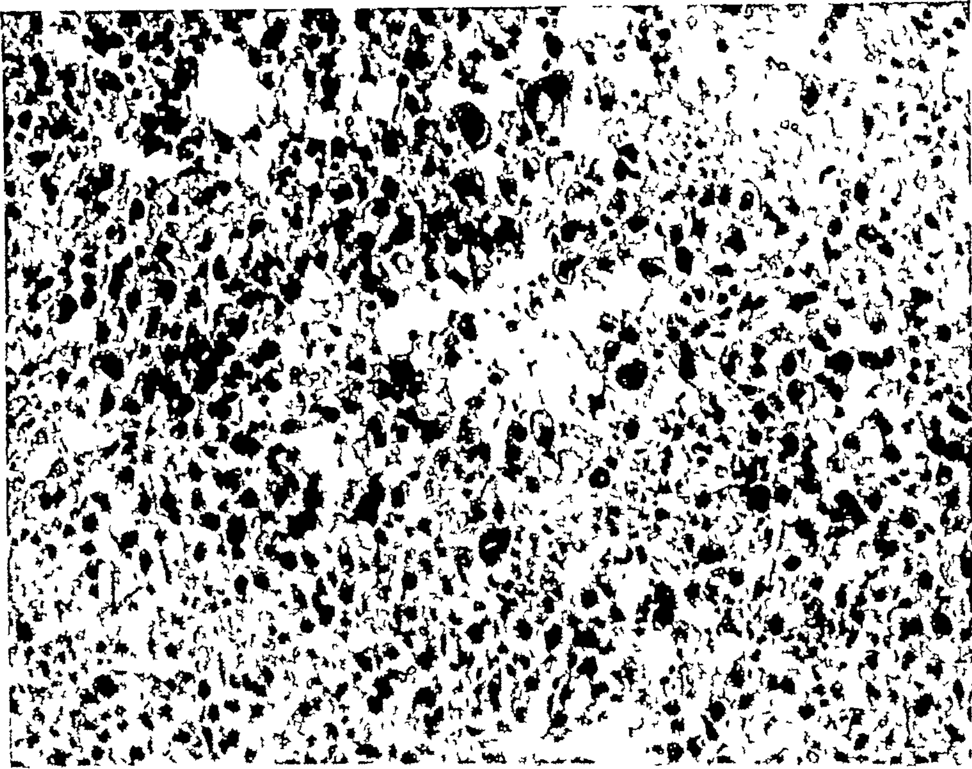


FIGURE 8 HEp 3 cell implants in normal recipient 86723 *Top* primary implant, day 7, sheet of cancer cells, some with nuclear pyknosis, with infiltration by polymorphonuclear and lymphoid cells (graded ++) *Bottom* repeat implant on day 7, a few scattered degenerating cancer cells in edematous acute inflammatory tissue (graded +)



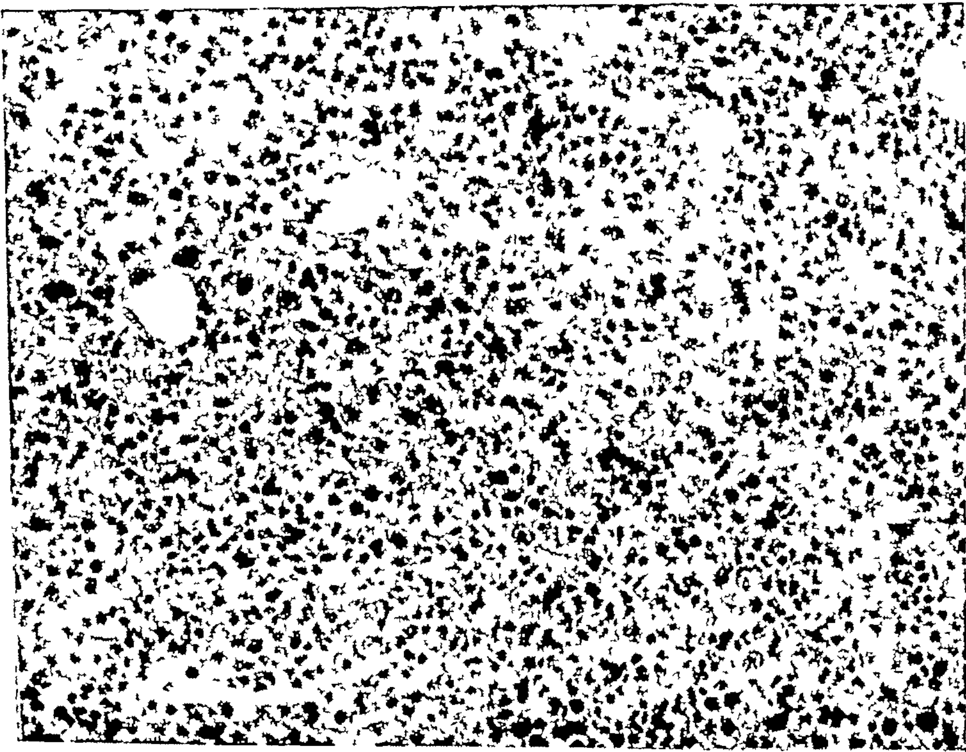


FIGURE 9 HEp 3 cell implants in normal recipient 99118 *Top* primary implant on day 7, numerous cancer cells, many with swollen nucleoli or pyknotic nuclei, edema, and inflammatory reaction (graded ++) *Bottom* repeat implant on day 7, few cancer cells enmeshed in reactive tissue consisting largely of polymorphonuclear cells (graded +)

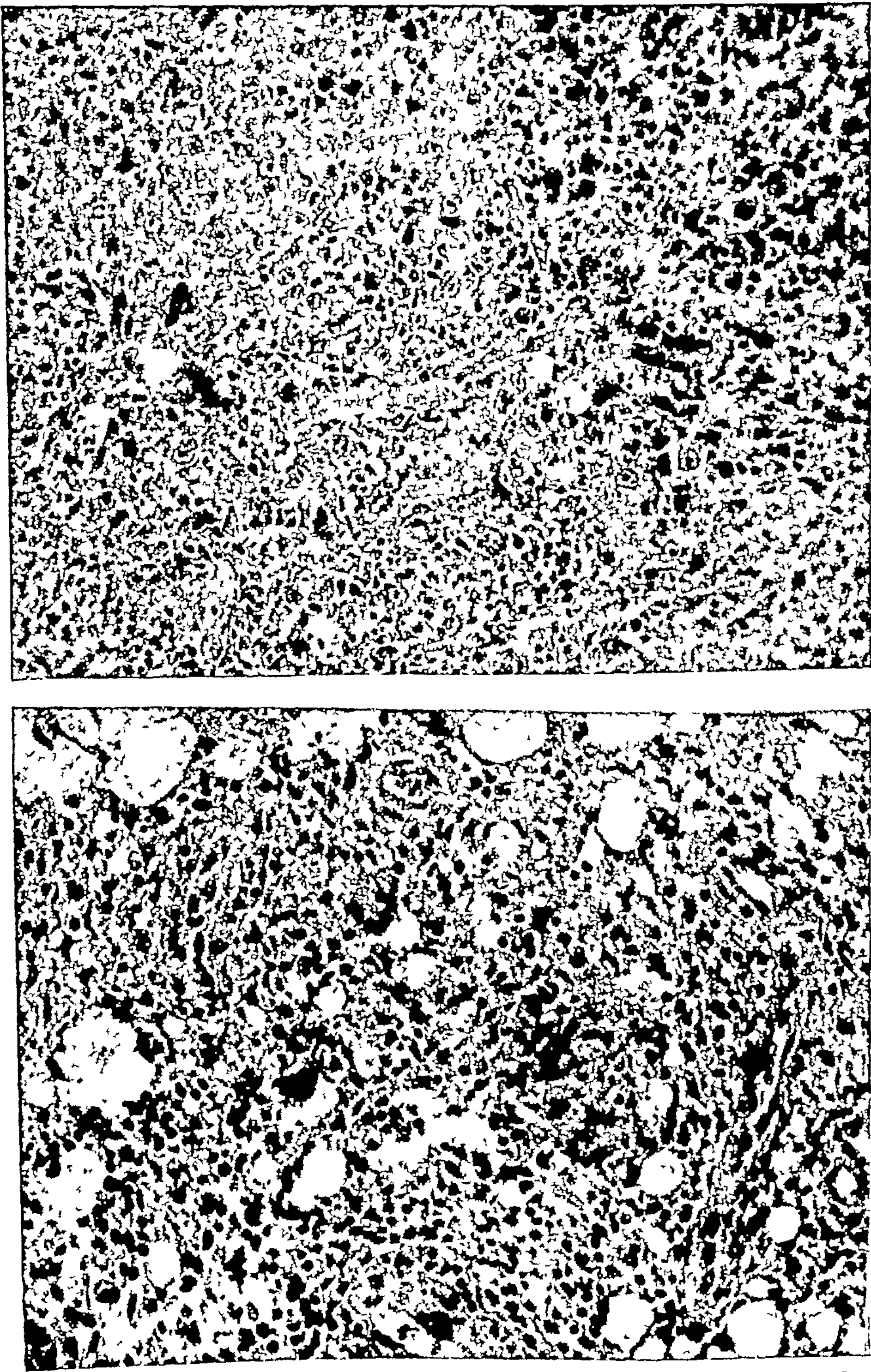


FIGURE 10 HEp 3 cell implants in normal recipient 98734 *Top* primary implant on day 14, strands of cancer cells with infiltration of polymorphonuclear and lymphoid cells (graded ++) *Bottom* repeat implant, day 14, connective tissue proliferation and lymphoid infiltration at implant site, no cancer cells (graded 0)

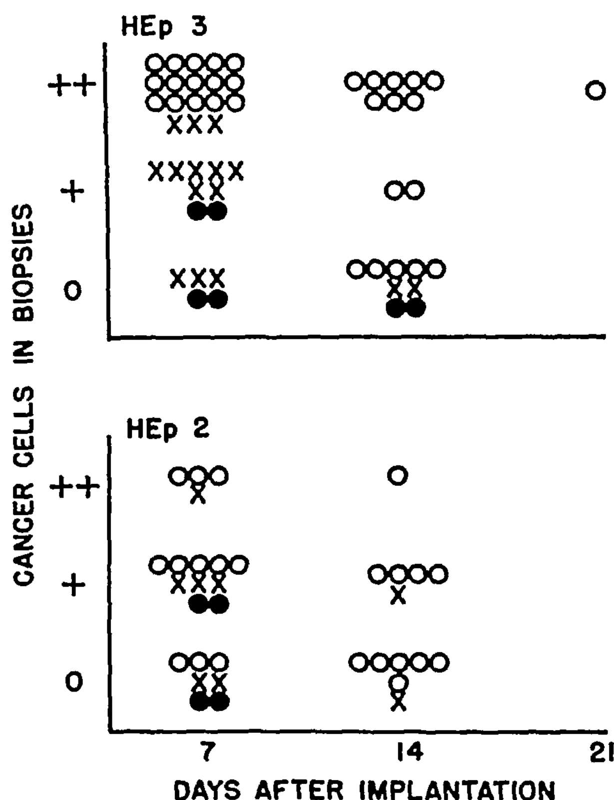



FIGURE 11 Graphic comparison of histological findings in biopsies of cancer cell implants in normal volunteers Open circles indicate primary implants Black dots indicate repeat implants in persons previously inoculated with same cell line Crosses indicate repeat implants in persons previously injected with different cancer cell lines Scheme for grading cancer cell growth is explained in text and illustrated in FIGURES 8, 9, and 10

in which none of the transplanted cancer cells can be found is graded as 0 Such nodules contained nothing but inflammatory tissue, or sometimes included areas of completely necrotic cells, which probably represented the inoculum If scattered cells or small nests of cells of the type inoculated could be identified the biopsy was graded as +, even if the cells showed definite degenerative changes If cancer cells were readily identified in considerable numbers (usually in small nests and sheets), but with inflammatory reactive tissue making up the major portion of the nodule, the grade was ++ (The grades +++ and ++++ are used for lesions consisting almost entirely of cancer cells, and with no acute inflammatory reaction Such lesions were never seen in the normal recipients ) The biopsies of HEp 3 cells 7 days after primary implantation all contained considerable numbers of the implanted cancer cells (grade ++) whereas 2 of the 4 biopsies taken 7 days after a second implant of HEp 3 cells showed no cancer cells The biopsies of HEp 3 implants given after previous implants of other cancer cell types generally contained only scattered cancer cells (grade +) and sometimes none At 14 days two thirds of the primary HEp 3 implants still contained the implanted cancer cells (grade + or ++) The two nodules biopsied 14 days after a second implant of HEp 3 cells did not contain cancer cells, nor did the two biopsies of HEp 3 implants that followed implants of other cancer cell types The biopsy data from HEp 2

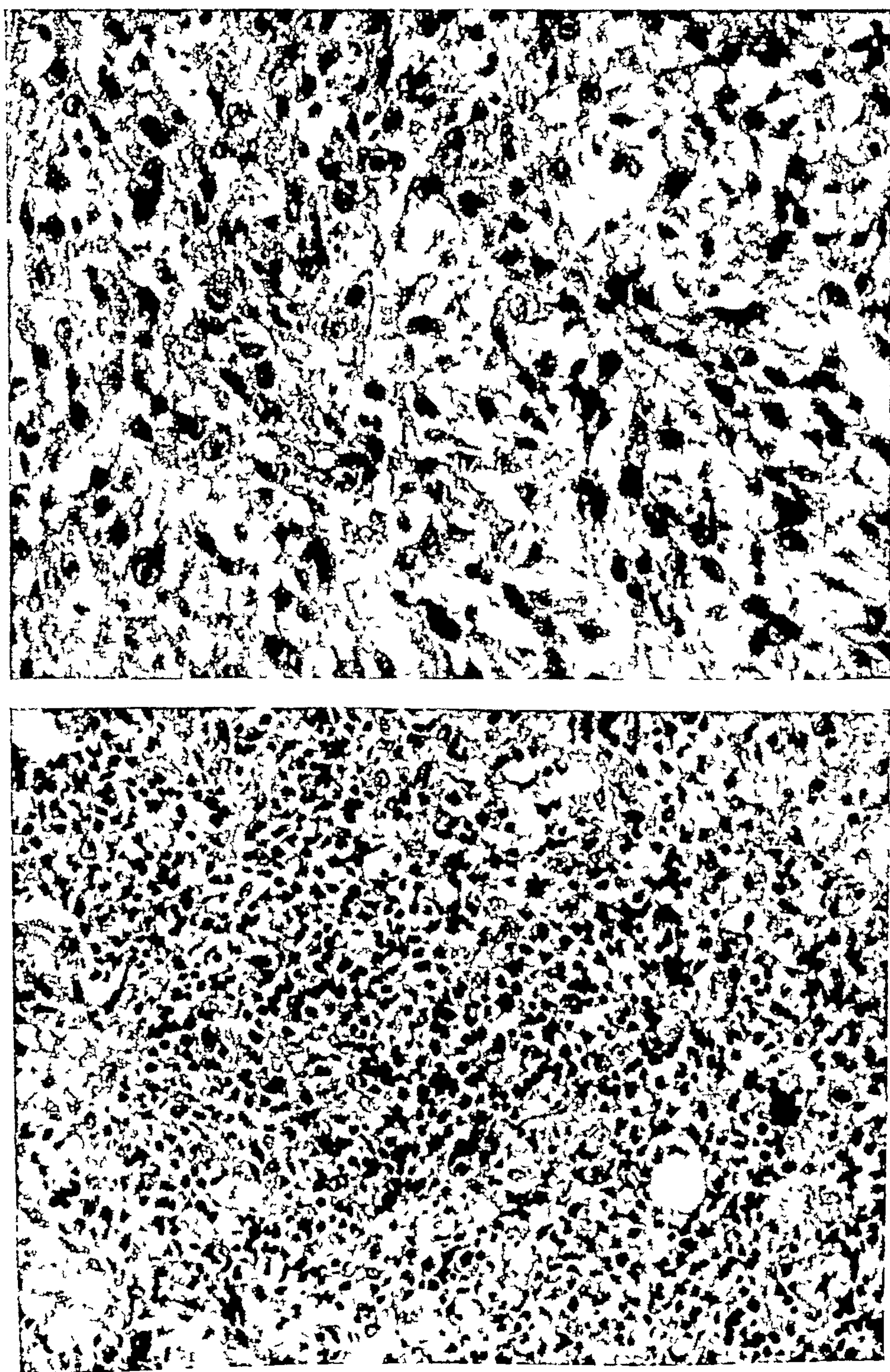


FIGURE 12. HEp 3 cell implants in patients with advanced cancer. *Top*: primary implant in patient JD54M on day 8; healthy-appearing cancer cells are scattered through subcutaneous connective tissue; there is no inflammatory reaction. *Bottom*: day-8 biopsy of HEp 3 implant, inoculated simultaneously with that above, in patient GB53M, who had previously had growth and rejection of HEp 2 cells; cancer cells rare; intense lymphoid infiltration.

implants reflect the lower growth potential of the cell on homotransplantation by both the lower frequency of positive biopsies and the smaller total number of biopsies, but in general show the same pattern as the HEp 3 biopsies

These histological data are consistent with the conclusion that normal persons who have received and rejected one cancer cell implant acquire the capacity for accelerated rejection of subsequent implants of the same cell type and probably other cancer cells of the types under study

*Cancer patients* Histological comparison of simultaneously inoculated and simultaneously biopsied material was possible in only one study of induced immunity in the cancer patients This is illustrated in FIGURE 12, which shows apparent rejection of the HEp 3 implant in a recipient previously inoculated with HEp 2 and unopposed growth of the simultaneous primary HEp 3 implant

### *Tissue Culture Data*

The recoveries of the implanted cancer cells in tissue culture (confirmed by implantation into conditioned rats) from biopsies of primary and repeat implants in normal recipients are presented in TABLE 1 The frequency of reisolation from day-7 biopsies is essentially identical for primary and repeat implants of all cell types except HEp 3 Even with HEp 3 the less frequent recovery of cancer cells from the repeat implants (8 of 13) as compared with

TABLE 1

REISOLATION IN TISSUE CULTURE OF CANCER CELLS FROM BIOPSIES OF HOMOTRANSPLANTS

Each entry refers to one biopsy specimen Fractions indicate number positive for cancer cells over total number cultured

| Cell type | Primary implants | Repeat implants | |
|---|---|---|---|
| | | Same cell previously | Different cell previously |
| **Day-7 Biopsies** | | | |
| HEp 1 | 1/3 | 2/3 | 1/1 |
| HEp 2 | 6/6 | 1/1 | 3/3 |
| HEp 3 | 6/7 | 0/1 | 8/12 |
| HeLa | 3/4 | 1/1 | — |
| HS 1 | 2/3 | 2/2 | — |
| J 111 | 1/1 | 0/1 | — |
| All types | 19/24 | 6/9 | 12/16 |
| Percentage | 79 | 67 | 75 |
| **Day-14 Biopsies** | | | |
| HEp 1 | 4/6 | — | — |
| HEp 2 | 3/7 | — | 0/2 |
| HEp 3 | 6/10 | — | 0/1 |
| HeLa | 1/5 | 0/1 | — |
| HS 1 | 3/4 | — | — |
| J 111 | — | — | — |
| All types | 17/32 | 0/1 | 0/3 |
| Percentage | 53 | 0 | 0 |

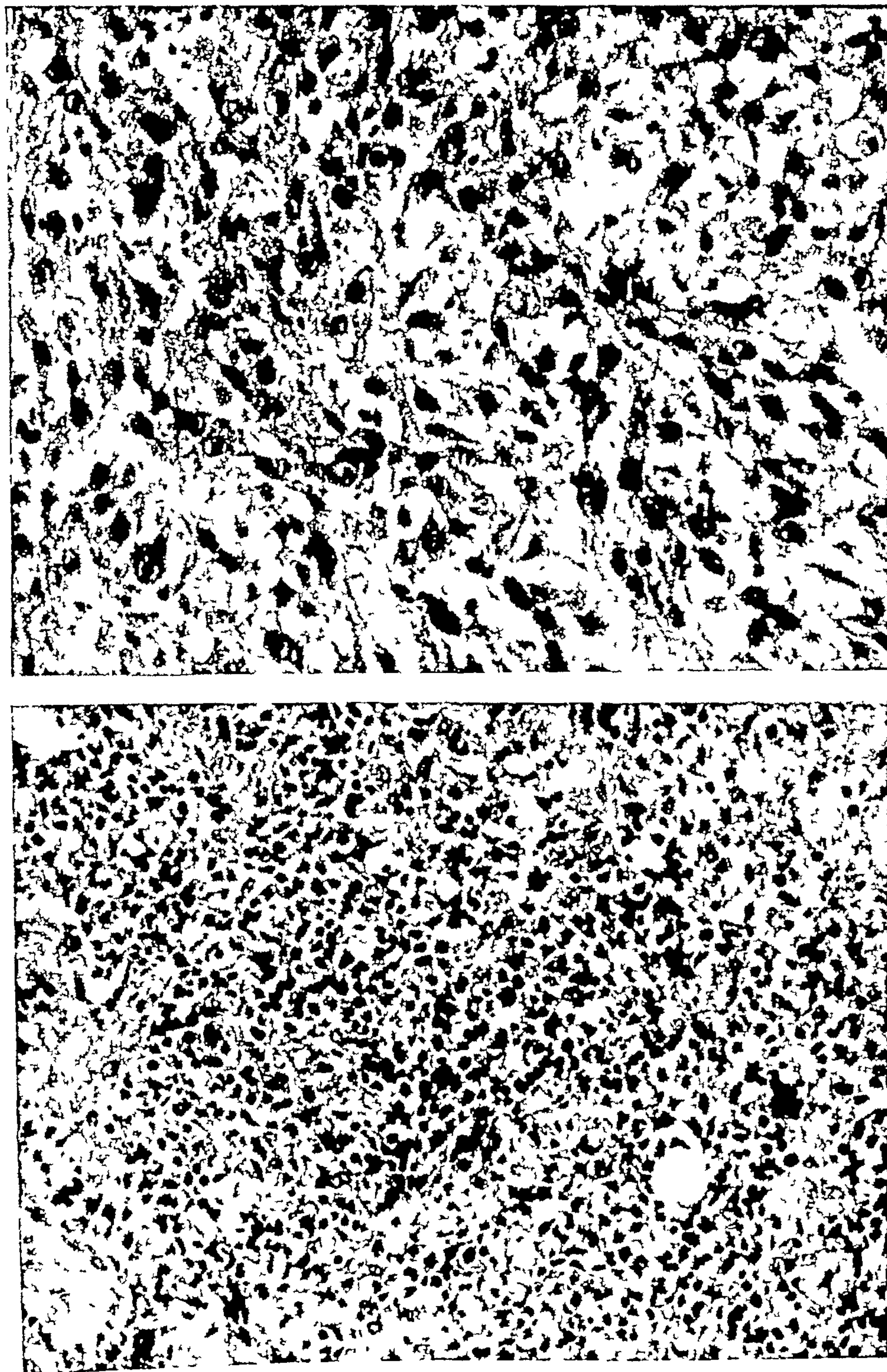


FIGURE 12 HEp 3 cell implants in patients with advanced cancer *Top* primary implant in patient JD54M on day 8, healthy-appearing cancer cells are scattered through subcutaneous connective tissue, there is no inflammatory reaction *Bottom* day-8 biopsy of HEp 3 implant, inoculated simultaneously with that above, in patient GB53M, who had previously had growth and rejection of HEp 2 cells, cancer cells rare, intense lymphoid infiltration

capable of producing specific antibodies to live-virus antigens in what appears to be a normal rate and quantity

*Summary*

It has been demonstrated that inoculation and rejection of an implant of cultivated human neoplastic cells produce in the recipient a subsequent state of induced immunity to the same and certain other cultivated lines of neoplastic cells, as judged by the size, persistence, and histological composition of the nodules that develop following such implantation. This immunity was induced by killed cancer cells as well as by living ones It was not induced by normal fibroblasts or amnion cells in the limited studies that have been performed to date This immunity was systemic and not limited to the site of previous implantation It follows that the neoplastic cells thus far studied are antigenic in a homologous host and that they contain some antigenic components in common, but are apparently different from the antigenic components of normal cells *In vitro* serologic studies have not yet given any convincing evidence of the production of circulating antibodies against the homotransplanted cancer cells

Cancer patients developed an induced immune reaction to these cancer cell implants, even though delayed rejection of the primary implants indicated marked impairment of their natural defense mechanisms

*Acknowledgments*

The authors express their indebtedness to the many persons who, with no expectation of personal gain, voluntarily acted as recipients of these homografts They are also greatly indebted to C A Doan and A G James of Ohio State University, Columbus, Ohio, to R H Brooks of Ohio State Penitentiary, Columbus, Ohio, and to C P Rhoads, R C Mellors, and R W Alvis of the Sloan-Kettering Institute for Cancer Research.

*References*

1 Southam, C M, A E Moore & C P Rhoads 1956 Homotransplantation of human cell lines Science **125** 158–160

2 Southam, C M, A E Moore, C A Doan, R H Brooks, A G James & C P Rhoads 1958 Homotransplantation of human cancer and normal cell lines into normal and cancer-patient volunteers In preparation

3 Moore, A E, L Sabachewsky & H W Toolan 1955 Culture characteristics of four permanent lines of human cancer cells Cancer Research **15:** 598–602

*Discussion of the Paper*

James T Grace, Jr, (*Roswell Park Memorial Institute, Buffalo, N. Y*) We have followed these studies of Southam and Moore with great interest

Their initial report[1] raised several fundamental questions First, could one relate growth of the tumor cells in the cancer patients wholly to depressed homograft defenses in these patients, or might properties inherent in malignant cells, but absent in normal cells, play a role? The failure of tumor cells to grow in normal volunteers suggested the former, while the failure of normal fibroblasts to grow in cancer patients suggested the latter Second, what

primary implants (6 of 7) cannot be accepted as a significant difference At 14 days cancer cells were not recovered from any of the 4 repeat implants that were cultivated, whereas one half of the primary implants were positive Little weight can be attached to these data because of the small number of repeat biopsies, but nevertheless it is of interest that the results are consistent with those obtained by nodule size and histological criteria

Correlation of these tissue culture and histological data reveals a 71 per cent concurrence of results (accepting both + and ++ histological grades as positive) Fourteen of the 83 biopsies studied by both techniques contained cancer cells on histological examination, but failed to yield the cancer cells in culture Ten showed the reverse discrepancy, that is, cancer cells isolated in culture but not present in the histological sections

### *Antibody Studies*

Complement fixation studies were negative using a HEp 2 cell antigen and serial specimens from 20 recipients (18 normals and 2 cancer patients) who had received one or more implants of HEp 2 cells No conclusion is possible, however, because without a known positive antiserum there is no proof that the antigen was adequate

The Ouchterlony agar-diffusion technique (studies by Leonhard Korngold) likewise gave negative results with HEp 2 and HEp 3 antigens and serial serum specimens from 6 normal recipients

Serial serum specimens from 13 recipients (10 normals and 3 cancer patients) were tested for inhibitory effect against HEp 3 by Helene Toolan, using *in vitro* incubation and conditioned rats as test host Although several sera were inhibitory for HEp 3 by this technique, there were apparent inconsistencies in the time of appearance of cytotoxicity, and cytotoxicity sometimes appeared in recipients whose only implants were cell types other than HEp 3 Thus no conclusion was possible from these studies concerning the production of serum cytotoxins

The search for antibodylike cytotoxic activity using tissue cultures was only slightly more encouraging Serial serum specimens, collected from 12 normal recipients from just before until 7 weeks after implantation, were tested against 5 lines of normal and neoplastic human cells Several recipients showed a progressive development of cytotoxicity against one or another cell types that might be consistent with production of antibody This effect, however, was not specific for the cell type inoculated, but frequently affected other cell types as well The frequency with which cytotoxic effects (presumably nonspecific) were seen even in preinoculation control sera makes it impossible to be certain from the amount of data now available that the appearance of cytotoxicity after inoculation was not due to pure chance The results are somewhat suggestive, however, and merit further investigation in future studies where it may be possible to control better the collection of sera so as to avoid nonspecific toxicity

Although no convincing demonstration of anticancer-cell antibodies has yet been possible, it has been repeatedly demonstrated that these patients are

was related to rejection of the stromal elements and that, since the tumor and skin had the same stromal elements, parallelism of rejection was anticipated This assumption is not completely warranted, since we know that experimental tumors carried in conditioned heterologous or homologous hosts may be transplanted successfully back to the strain of origin In such cases the stromal elements from the foreign hosts obviously do not persist, yet the tumor cells survive and form new stroma

I congratulate Southam and Moore on their important and stimulating work

*Reference*

1 SOUTHAM, C M , A E MOORE & C P RHOADS 1957 Homotransplantation of human cell lines Science **125** 158–160

effect did propagation in tissue culture or heterologous hosts have on the growth potential of these cells? In other words, might one expect this type of aggressive behavior with homografts of any human tumor? This could be fundamentally important to our understanding of the malignant process in man

In order to investigate some of these factors we elected to do simultaneous direct grafts of both malignant tumor and normal skin from the same donor The recipients were volunteer patients with advanced malignant disease It was reasoned that, if host factors were largely responsible for growth or rejection of the homografts, one might expect persistence of the normal skin to parallel growth of the tumor However, if properties peculiar to the tumor were involved in its growth, earlier rejection of the skin should be observed

Fresh tumors were used to compare the growth of such cells with the serially propagated lines of Southam and Moore These included malignant melanoma, Hodgkin's disease, carcinomas of the breast, ovary, stomach, colon, and urinary bladder All were implanted as 1- to 2-mm fragments subcutaneously

All tumor and skin grafts showed initial clinical takes Histological control was utilized for confirmation The tumor nodules increased in size steadily as long as the skin grafts remained healthy When the skin showed early rejection changes the tumor nodules stopped growing When total skin rejection occurred the tumor nodules softened and regressed In no instance did a tumor nodule continue to grow after total rejection of the skin graft In only one instance did the tumor nodule regress while the skin graft remained healthy

In 20 studies the skin grafts persisted for an average of 16 days, this was almost exactly paralleled by the tumor implants In one subsequent study a skin graft persisted for 60 days The corresponding tumor nodule started regressing at 24 days and was no longer palpable at 35 days

In 2 cases second grafts of skin and tumor from the original donors were done, and accelerated rejection of both repeat grafts was observed In 4 cases tumor implantation was done 5 days prior to skin grafting, and in all cases rejection of the skin of the tumor donor preceded that of a control skin graft from an unrelated donor

Thus, under the conditions of this experiment, persistence of normal skin closely paralleled growth of the malignant tumors Since the skin persisted somewhat longer than would be expected in normal persons, it appears that some depression of homograft defenses is present in these patients The second-set responses and eventual rejection of all homografts attest to the quantitative nature of this depression

The failure of the fresh tumor implants to show the aggressive patterns of growth observed with the cell lines of Southam and Moore suggests that serial propagation either enhanced the virulence of their cells, or the fact that the cells could be serially carried showed increased virulence of the original cells, or both It would be most interesting to compare the antigenic properties of the present cells with the antigenic properties of the original cells

It was suggested that rejection of the fresh tumor implants in our experiments

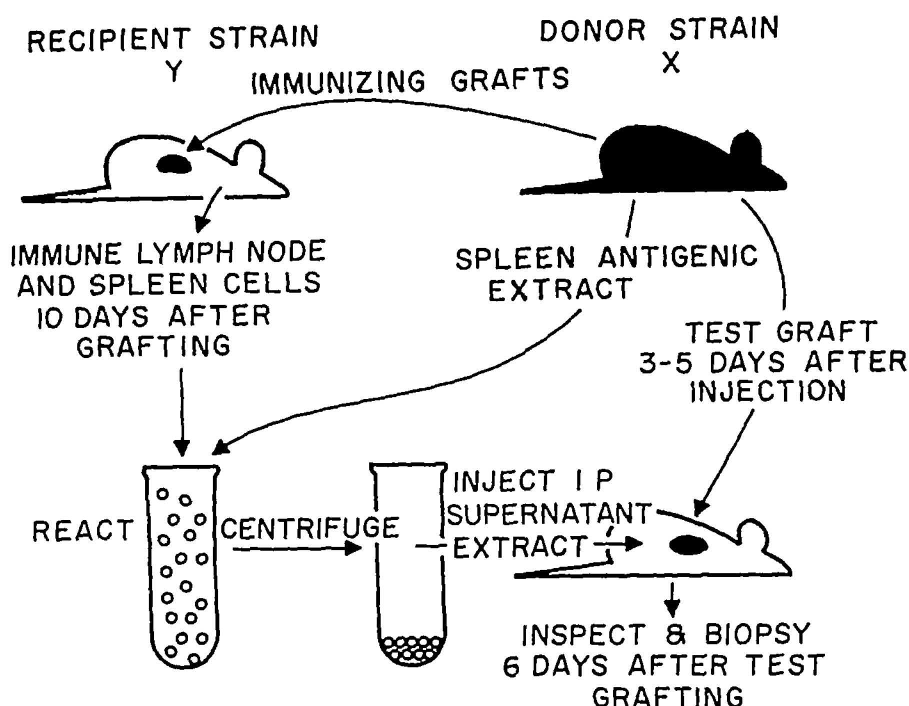



FIGURE 1 Design of a typical experiment

Two of these, A/Jax and CBA/Jax, differ by at least 15 independently segregating genes (Barnes and Krohn, 1957) whereas the others, C57BL/10 and C57BL/10 D2, are virtually coisogenic except for alleles at the single locus H-2 (Snell and Borges, 1953) For convenience, these latter strains will here be designated B and D, respectively

The technique of skin grafting was identical with that of Billingham and Medawar (1951) Methods for the evaluation of graft survivial, the determination of median survival times, and the regional immunization of mice have all been fully described elsewhere (Billingham *et al*, 1954a, Billingham *et al*, 1954b) and will therefore be mentioned only in the briefest detail

The median survival times (MST) of "first-set" skin homografts in the strain combinations used by us were CBA → A 10 days, A → CBA 11 days, B ↔ D 9 days Unless transplanted to mice previously immunized either by skin grafts or antigenic extracts, the test grafts never showed even the slightest epithelial destruction when examined histologically on the sixth day after transplantation On the other hand, grafts on recipient mice that had previously been injected with antigenic extracts of donor-strain origin as a rule displayed unmistakable signs of epithelial breakdown, the extent of which may be accepted as a measure of the relative strength of the antigenic potency of the extract (see Billingham *et al*, 1956) This biological test was therefore used in order to compare the antigenicity of homologous tissue extracts before and after contact with lymphoid cells removed from either specifically immune or normal mice

The "immune lymph node" donors were immunized by the transplantation of a skin homograft to each side of the chest, thus permitting a maximal harvest

# CELL-BOUND ANTIBODIES IN TRANSPLANTATION IMMUNITY*

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## *Introduction*

Although there is no convincing evidence that serum antibodies cause the destruction of solid tissue homografts such as transplanted skin, there can be little doubt of the crucial role played by host cells For example, Mitchison (1953, 1954) as well as Billingham *et al* (1954a), have shown that the regional lymph nodes and spleens of specifically immunized mice are capable of transferring immunity to normal mice This phenomenon is known as "adoptive immunity" because it has been demonstrated that it depended upon the transfer of sensitized cells capable of survival and continued reactivity in the hosts rather than on the transfer of preformed antibodies Nevertheless, these experiments do not entirely rule out the possibility that, at the time of transfer, the cells already had specific reactive sites within them or on their surfaces The recent findings of Billingham *et al* (1956) that homologous transplantation antigens can be extracted in solution from mouse tissues has made it possible to investigate this problem by direct *in vitro* experiments

The design of a typical experiment was as follows (FIGURE 1) Antigenic extract was prepared from normal spleens of the donor strain (for example, strain X) Immune cells were obtained from the lymphoid tissues of recipient strain (for example, strain Y) mice that had reacted against X-skin homografts Normal (nonimmune) cells were simply prepared from the lymphoid tissues of normal Y-strain mice Following its exposure to either normal or immune cells the extract was tested for antigenicity by injection into normal Y recipients A third group of Y mice received an equal volume of X extract that had not been exposed to either immune or nonimmune cells All three groups of recipients were subsequently challenged with X-skin homografts, if the immunity elicited by the extract treated with immune cells was appreciably feebler than that elicited by the others, then this was taken as evidence that the cells had been instrumental in removing or inactivating some or all of the antigenic molecules originally present It will be shown that these essentially *in vitro* experiments reveal a clear-cut difference in the behavior of immune and normal cells—a difference that is compatible with the concept of the existence of cell-bound antibodies in transplantation immunity

## *Materials and Methods*

The mice used in these experiments belonged to 4 highly inbred strains obtained from the Roscoe B Jackson Memorial Laboratory, Bar Harbor, Me

* The opinions or assertions contained in this paper are those of the writers and are not to be construed as official, or as reflecting the views of the Navy Department or of the naval service at large

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centrifugation (2500 rpm for 10 minutes), and the clear supernatant was then ready for use. The temperature of the antigenic extracts was kept at about 5° C. throughout the experiments.

*Preparation of the Cell Suspensions*

Both immune and normal cell suspensions, obtained from lymph nodes and spleens, were prepared as follows. The tissues were expressed into normal citrate saline (NCS) composed of 9 parts 0.15 M NaCl and 1 part 0.15 M sodium citrate, and the cells were dispersed by very gentle pipetting. Large cell clumps were allowed to settle out and were excluded. In some experiments the number of cells per ml. was established by a cell count, so that the same number of normal and immune cells could be used for the absorption of equal volumes of the antigenic extract; in others, this was achieved rather less accurately by adjusting the volumes of the sedimented cells. However, in either case all cell suspensions were washed once and, in order not to add appreciably to the volume of the matter to be injected, the sedimented cells were immediately suspended in the corresponding antigenic extracts. The cells were chilled throughout the experiment.

*Conduct of Experiments*

The design of the experiments (FIGURE 1), although relatively simple, had certain practical limitations, the most important of which concerned the amount of tissue required for the preparation of extracts and cell suspensions.

On the average, 2½ spleens provided an extract of sufficient strength to immunize a single mouse recipient and, for the absorption of such a dose, the cells from 12 lymph nodes and 3 spleens were used. This heavy demand on valuable inbred stocks explains the rather small number of mice involved in the individual experiments.

Antigenic extracts and cell suspensions were usually prepared simultaneously so that the materials could be used in as fresh a state as possible. The extract was divided into 3 equal parts: the first was added to the sedimented immune cells, the second to the nonimmune cells, and the third was reserved as a straight control without cellular absorption. The cells were suspended in the extracts by careful and gentle pipetting and exposed to the extract for 90 minutes at 5° C., all 3 tubes being gently agitated with the aid of a mechanical shaker. The cells were spun down at 3500 rpm for 10 minutes and the clear supernatants carefully removed. Centrifugation was repeated to ensure that these were entirely cell free, but it is perhaps relevant to point out that, even if cellular contamination had occurred on any scale, this would have weighed the experiment against our hypothesis insofar as the immune (and not the normal) cells might have immunized their hosts adoptively. Finally, the antigenic extracts of each category were injected intraperitoneally, in equal amounts, into the homologous recipients. Each thus received an equal volume of extract, no matter whether this had been treated with immune or nonimmune cells or whether it had not been treated at all.

Three to 5 days after injection, test grafts from mice of the antigen donor

of 4 regional nodes per mouse The nodes were removed 10 days after skin grafting, that is, on or very near to the median survival time (MST) of the immunizing grafts The test grafts of mice injected with the antigenic extracts were invariably inspected 6 days after their transplantation and careful note was taken of such features as soundness of healing, vascularization, and obvious outward signs of epidermal destruction They were then removed *in toto* and prepared for microscopic examination The system of scoring (discussed by Billingham *et al*, 1954a) was based on the degree of survival of the epithelial components of the graft Degrees of survival (in per cent) were classified in the following way

| | |
|---|---|
| 100 | Complete survival |
| 75 | Breakdown in progress |
| 50 | Breakdown in progress |
| 25 | Breakdown in progress |
| 0 | Breakdown complete |
| <0 | Breakdown complete, with maximal immunity |

The last category, <0, suggesting a minus quantity of survival, was intended to single out a grouping of grafts in which breakdown must have been long complete, as revealed by the almost complete absence of vascularization, the extreme weakness of attachment to the host beds, and the death and disruption of the dermal as well as the epidermal components The significance of this additional category will emerge from the discussion

*Preparation of Antigenic Extracts*

Aqueous antigenic extracts were prepared by the method previously described by Billingham *et al* (1956) Spleens were excised from their normal donors, weighed, and pressed through a tissue sieve into a buffered normal saline (BNS), composed of 9 parts 0 15 NaCl and 1 part 0 1 M sodium phosphate buffer, *p*H 7 4 The cell suspension was washed once, and the sediment was resuspended in normal saline to which were then added 3 volumes of water The rather swollen cell mass was again spun down and the supernatant discarded The sediment was then dispersed in water by prolonged and vigorous pipetting, the volume of distilled water depending both upon the desired concentration of the final antigenic extract and the number of mouse recipients involved (in practice, the volume was always limited to a maximum of 2 ml per recipient)

The resulting suspension was homogenized either by exposure to the ultrasonic vibration of a 20 kc/sec magnetostriction transducer,* or by blending with a Virtis 45 homogenizer† at top speed (45,000 rpm) for 3 minutes The time of exposure to ultrasound varied somewhat because of unpredictable variability in the power output of the instrument, the average being about 120 seconds The end point was judged from the clarity of the material and the number of intact cell nuclei present in samples withdrawn at regular intervals The homogenate was spun at 2500 rpm and the salt content of the supernatant restored to 0 15 M The precipitate that formed promptly was removed by

* We thank A Ellis of the California Institute of Technology for permitting us to use an apparatus of his own construction

† Product of the Virtis Co, Yonkers, N Y

a finite number of cells being capable of reacting with only a finite number of antigenic units. From what is known of the relationship between antigenic dose and immune response in transplantation immunity (Medawar, 1944, Billingham *et al.*, 1957), it seemed likely that a considerable proportion of antigenic molecules had in fact been inactivated in these experiments. The following rather limited experiment was designed to test this interpretation.

Untreated antigenic extracts were prepared from the spleens of 24 donor mice and injected into 12 homologous animals at 3 different dosage levels. The scores of the test grafts 6 days after transplantation were as follows:

266 mg spleen extract per mouse, scores <0, <0, <0, <0
200 mg spleen extract per mouse, scores 0, 0, 0, –
133 mg spleen extract per mouse, scores 25, 25, 0, 0

One animal (–) failed to recover from anesthesia when skin-grafted. The difference between the <0 grafts on the one hand, and those scoring 0 and 25 on the other, was striking. Whereas the former were largely unattached to their beds, the latter had healed in with tolerable firmness. However, the point of special interest is here that halving of the antigenic dose led to a reduction of immunity almost exactly comparable with that brought about by the immune cells in experiments 5 and 6. Consequently, it seemed more than probable that, the low survival scores notwithstanding, the immune cells had absorbed at least half of the antigenic molecules originally present in the extracts. Furthermore, in experiment 4, 3 additional mice (not listed in the table) were injected with one half the standard antigenic dose for that experiment; their graft scores were very similar to those of the mice injected with extract absorbed with immune cells. Again there was an indication that less than one half of the antigenic activity remained after exposure to the immune cells.

Variability in the performance of the ultrasonic equipment unquestionably contributed to the differences in antigenic efficacy of various extracts; indeed, it was not surprising to produce extracts entirely devoid of antigenic activity. In such instances all test grafts showed complete survival at the usual time of biopsy, and the experiments were, of course, invalidated. Variability in the efficiency with which different cell suspensions appeared to inactivate the extracts might have depended partly upon this very same factor, that is, the strength of the extract to begin with, but it may be necessary to take into account also the varying degrees of damage sustained by the cells in the course of preparation, especially since the cells may have been rather susceptible to injury in the absence of calcium and magnesium ions.

It may be mentioned in passing that two experiments were set up to determine the ability of immune cells to transfer immunity adoptively *after* reaction with homologous antigenic extracts. They were found to be fully capable of doing so.

## *Discussion*

The experiments show clearly that, when a state of immunity is set up in response to the transplantation of skin homografts, cells of the regional lymph

TABLE 1

SURVIVAL SCORES OF SKIN HOMOGRAFTS SIX DAYS AFTER TRANSPLANTATION TO MICE PREVIOUSLY INJECTED WITH ANTIGENIC EXTRACTS, EITHER UNTREATED OR TREATED WITH NORMAL OR IMMUNE LYMPHOID CELLS

| Experiment No | Cells (vol , ml /dose) | Antigen (wet wt. spleen, mg /dose) | Survival scores (%) | | |
|---|---|---|---|---|---|
| | | | (1) Antigen control | Treated with cells | |
| | | | | (2) Nonimmune | (3) Immune |
| 1 | D | B | — | | |
| | ( 25) | (240) | — | 25 | 75 |
| | | | | 0 | 75 |
| 2 | B | D | 25 | 50 | 100 |
| | ( 15) | (215) | 0 | 0 | 100 |
| 3 | B | D | 0 | 25 | 100 |
| | ( 3) | (270) | 0 | 0 | 75 |
| 4 | CBA | A | 75 | 25 | 100 |
| | ( 25) | (225) | 25 | 25 | 100 |
| | | | 0 | 0 | 25 |
| 5 | B | D | <0 | <0 | 25 |
| | ( 15) | (260) | <0 | <0 | 25 |
| | | | <0 | <0 | 0 |
| | | | <0 | <0 | 0 |
| 6 | B | D | <0 | <0 | 50 |
| | ( 15) | (250) | <0 | <0 | 25 |

strain were transplanted to all recipients Six days later the grafts were inspected, biopsied for histological examination, and eventually assigned a score indicating their degree of epithelial survival

*Results*

TABLE 1 summarizes the 6 experiments A comparison of graft survival scores in columns 1 and 2 makes plain that the treatment of extracts with nonimmune cells did not significantly impair their antigenicity Furthermore, the combined data of these 2 categories show that, in general, the extracts were capable of inducing a very high degree of immunity in the hosts Only 2 of the 28 grafts (2/28) displayed even a moderately high degree of survival (scores 50, 75), and 20 of 28 (20/28) were without epithelial survival of any degree

By contrast, prior treatment of the extracts with immune cells appreciably lowered their antigenic activity Five of 15 grafts (5/15) gave no hint whatever of an immune state, of the remaining 10, 7 (7/15) had significantly higher scores than their controls, and only 3 (3/15) revealed an immunity which was indistinguishable from that of their corresponding controls (experiment 4, score 25, experiment 5, scores 0, 0)

Special significance is attached to the results of experiments 5 and 6 Both the untreated extracts and those "absorbed" with nonimmune cells elicited an immunity of the extreme kind denoted by the score <0 It therefore seemed very probable that what appears to be extremely feeble and unsatisfactory absorption by the immune cells was here due to a considerable antigen excess,

unlikely that, once in the graft, the antibodies are released locally, the host round cells in a graft undergoing destruction certainly do not all come into close physical contact with the graft epithelial cells, although they all undergo necrosis. It might be mentioned in passing that in the present *in vitro* experiments the appearance of the immune cells did not change after reaction with antigenic extract, nor was there evidence of cellular agglutination. This aspect is still being studied.

Relevant to these considerations may be the finding that the cell-bound antibodies either do not retain their activity or are unable to intervene effectively if their cellular vehicles are disrupted by ultrasonic methods. One of us (L. B., unpublished) has attempted to curtail the survival times of skin homografts through the agency of cell-free homogenates of immune lymph node and spleen cells. Repeated intraperitoneal injections of large doses had no discernible effect on the immunological response of the homologous hosts. Although it might be argued that the antibodies were simply unable to withstand exposure to ultrasound, it seems rather more likely that their ineffectiveness under these conditions was due to the fact that they were never given an opportunity of reaching the graft site.

The hypothesis most attractive to us, one that, moreover, receives considerable support from our present findings, is that the process of active immunization entails the formation of antibodylike reactive sites either on the surface of cells of regional lymphoid tissue or within them, and that it is these reactive sites that play the dominant role in the destruction of the transplant. The question of circulating serum antibodies has recently been adequately discussed (Billingham and Brent, 1956; also see the reviews of Gorer, 1956; Medawar, 1944; and Brent, 1958). Although it cannot be stated categorically that circulating serum antibodies such as hemagglutinins take no part whatever in the process, it does seem that, at least in the case of normal tissue grafts, their action (if any) can be only auxiliary. The role of complement in the destruction of homografts is equally in doubt.

## *Acknowledgments*

We thank R. D. Owen of the California Institute of Technology, who provided the facilities for this work, and P. B. Medawar of University College, London, for their interest and much valued criticism. Acknowledgment is due also to the Rockefeller Foundation, New York, N. Y., most of the work reported in this paper having been carried out while one of us (L. B.) held a Foundation Research Fellowship.

## *References*

ALGIRE, G. H., J. M. WEAVER & R. T. PREHN. 1954. J. Natl. Cancer Inst. **15**: 493.
BARNES, A. D. & P. L. KROHN. 1957. Proc. Roy. Soc. London **B146**: 505.
BILLINGHAM, R. E. & L. BRENT. 1956. Brit. J. Exptl. Pathol. **37**: 566.
BILLINGHAM, R. E., L. BRENT & P. B. MEDAWAR. 1954a. Proc. Roy. Soc. London **B143**: 58.
BILLINGHAM, R. E., L. BRENT & P. B. MEDAWAR. 1956. Nature **178**: 514.
BILLINGHAM, R. E., L. BRENT, P. B. MEDAWAR & E. M. SPARROW. 1954b. Proc. Roy. Soc. London **B143**: 43.
BILLINGHAM, R. E., L. Brent & N. A. MITCHISON. 1957. Brit. J. Exptl. Pathol. **38**: 467.

nodes and the spleen acquire the ability to react *in vitro* with the antigens responsible for the induction of immunity This ability is evidently not possessed by normal, nonimmune lymphoid cells It must be presumed that antibodylike reactive sites are created on or within the lymphoid cells and, in view of the general corpus of evidence against the existence of protective serum antibodies in this particular system (see below), these may be considered to be "cell-bound antibodies" Other and perhaps more circumstantial evidence in favor of the existence of this type of antibody in transplantation immunity can be drawn from previous investigations, which leaves little doubt as to its activity *in vivo*

For example, there is convincing evidence that the fountainhead of the immune response to first-set tissue homografts is chiefly confined to the regional lymph nodes and, to a lesser extent, the spleen (Mitchison, 1953, 1954, Billingham *et al*, 1954a) The more distantly located lymphoid tissue (for example, the contralateral lymph nodes) is relatively ineffective in transferring immunity adoptively Morphological studies have also shown that distant lymph nodes receive, at best, only a feeble antigenic stimulus from a tissue homograft (Scothorne and McGregor, 1955, Scothorne, 1957) It would therefore appear that the antigens issuing from the graft are caught and neutralized fairly efficiently by the regional lymphoid tissue This localization of antigens *in vivo* can be envisaged as the result of intervention by locally produced antibodies which, in the absence of circulating protective antibodies, are presumably fixed to the cells Very recent work on the guinea pig has also revealed an antigen-immune cell reaction shown by a delayed type of cutaneous lesion (Brent *et al*, unpublished data) Here immune (regional) lymph node cells, when injected intradermally into the donor whose skin grafts had been used for the immunization, produced local inflammatory wheals Again it may be presumed that antibodies associated with the cells reacted with the antigens of the surrounding host cells, and that since serum failed to react likewise, the antibodies remained cell bound

It is of some importance to consider briefly the possible relationship of the cell-bound reactive sites to those "antibodies" that implicitly overcome tissue homografts *in vivo* Our own data indicate that reactive cells are present in the regional lymphoid centers, but they do not prove that they appear at the graft site during the process of tissue destruction Do these cells or their descendants actually find their way into the homograft?

Perhaps the best evidence for the necessity of cellular invasion as a prerequisite for graft rejection comes from the observations involving diffusion chambers (Algire *et al*, 1954, Weaver *et al*, 1955) It has been firmly established that homografts are not destroyed, even within immune hosts, so long as they are physically protected from invasion by host cells On the other hand, washed immune spleen cells included within the chambers bring about rapid destruction of the target cells even when the hosts are isologous with the latter It can be concluded from experiments of this kind that the cell-bound antibodies are not released into the general circulation in operative concentrations, but remain on or within the cells until they combine with their corresponding antigens within the graft Although this must remain pure conjecture it is not

# HISTOCOMPATIBILITY IN INBRED STRAINS OF GUINEA PIGS

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## Introduction

The desirability of employing inbred strains of animals with a high degree of genetic identity for studies of tissue transplantation is well recognized. The goal of achieving genetic identity to the degree of complete histocompatibility has been realized only in several strains of mice and, to a lesser extent, in rats. The guinea pig has been used comparatively infrequently in transplantation studies, and the available studies suffer considerably from the lack of adequately inbred strains and consequent inconsistent experimental results.[1, 2] Although occasional allusions to inbred strains of guinea pigs appear in the literature, no evidence of histocompatibility has been presented.[3] Consequently, it appeared desirable to evaluate the degree of histocompatibility within (as well as between) each of the two strains of inbred guinea pigs available to us.

### *Experimental Animals*

The pedigree of our guinea pigs can be traced back to 1906 when Wright[4] initiated a genetic investigation of the effects of prolonged, strict brother-sister inbreeding on such qualities as vigor, fertility, and growth of the resulting lines of animals. Starting with 35 pairs of guinea pigs, the surviving breeding lines diminished over the ensuing years for a number of reasons and, in 1917, all but 5 of the remaining 12 strains were destroyed. Of these 5 lines, 2 strains, namely Strain 2 and Strain 13, have survived to the present, perpetuated through approximately 50 generations of strict brother-sister matings.

The progress of inbreeding in guinea pigs, from heterogenous stock to genetic homogeneity, can profitably be ascertained through the studies of Loeb[5] with these same strains of animals. In a series of experiments in 1927, and again in 1931, Loeb evaluated the degree of histocompatibility through the use of a variety of subcutaneously implanted organ fragments. After 17 to 25 generations of consecutive brother-sister matings, nearly autogenous compatibility was observed within Strain 2, while Strain 13 showed slightly less favorable compatibility, with greatly delayed graft destruction. Thus, even though genetic identity had not been attained following about 20 generations of sibling inbreeding, genetic homogeneity, as judged by histocompatibility, was evidently closely approached. It will be shown below that both strains of guinea pigs have now attained complete intrastrain histocompatibility, but it is interesting to note that the multicolored coats of these animals (white, black, and brown) show no uniformity in pattern nor distribution of coloration within either strain, but differ widely among individuals despite the high degree of genetic homogeneity.

BILLINGHAM, R E & P B MEDAWAR 1951 J Exptl Biol 28 385
BRENT, L 1958 Progr in Allergy In press
GORER, P. A 1956 Advances in Cancer Research 4: 149
MEDAWAR, P B 1944 J Anat 78· 176
MITCHISON, N A 1953 Nature 171. 267
MITCHISON, N A 1954 Proc Roy. Soc London B142: 72
SCOTHORNE, R J & I A McGREGOR 1955 J Anat 89. 283
SCOTHORNE, R J 1957 Ann N Y Acad Sci 64(5). 1028
SNELL, G D & P R F BORGES 1953 J Natl Cancer Inst 14. 481
WEAVER, J M , G H ALGIRE & R T PREHN 1955 J Natl Cancer Inst 15 1737

All grafts were uniformly cut at a thickness of 20/1000 of an inch (508 $\mu$), which corresponds to about one third skin-thickness grafts (FIGURE 1b) All graft beds were cut to a depth of 40 to 50/1000 of an inch (1016 to 1270 $\mu$) This depth closely approximates full thickness of skin under the conditions of these experiments Since guinea pigs do not undergo skin cycles with the concomitant cyclic fluctuations of skin thickness, complete skin excision is reliably attainable if the size, age, and anatomical skin area of the animals are held constant The combination of a split-thickness graft on a full skin-depth bed was selected in an attempt to provide optimal conditions for graft takes while still obtaining a satisfactory fur-bearing graft

Although grafts as large as 12 × 16 cm can be easily taken from and transplanted onto mature guinea pigs, 4 × 6 cm grafts were used for the present experiments It is considered important to allow an overlapping graft margin around the entire circumference of the graft to project beyond the edge of the bed onto which the graft is laid This precaution seals off raw surfaces most rapidly, effectively prevents extraneous infection, and facilitates the healing of edges with minimal scar formation To this end, grafts cut to a size of 4 × 6 cm were laid onto beds measuring 3 × 5 cm The uniformity of all dimensions of both graft and bed, achieved with this technique employing a dermatome, yields highly reproducible results and allows meaningful comparison of identical grafts under otherwise varied experimental conditions

All donor and recipient guinea pigs were between 2 and 4 months old at the first grafting and weighed between 250 to 400 gm Preparatory to grafting, the animals were anesthetized by an intraperitoneal injection of pentobarbital sodium (Nembutal) in the amount of 25 to 30 mg /kg The fur was clipped over both lateral thoracoabdominal aspects, and the remaining hair stubbles were removed by a 2 to 3 min application of depilatory lotion (Nair*), followed by thorough rinsing with water Asepsis was maintained by scrubbing the skin with pHisoHex† and aqueous Zephiran† and steam sterilization of all instruments and implements used in the operative field The skin was sponged with ether, and rubber cement was then applied to an area of the desired dimensions After cutting the graft the tissue was removed from the dermatome drum by grasping its periphery only It was then placed in 1 or 2 ml of sterile saline in a Petri dish until such time (not exceeding 30 min ) as it was applied to its recipient bed If 10 to 15 min are allowed to elapse between preparation of the bed and application of the graft, all capillary oozing ceases and the partially dried bed, sponged free of clotted blood, favors the adhesion of the graft to its bed If firm but gentle pressure is then applied for a few seconds, no sutures or other means of fastening the graft were necessary, as the coagulating plasma exudate rapidly fixes the graft into place Finally, dry sterile gauze was laid over the graft and a circumferential adhesive tape dressing was applied loosely for protection from external trauma The dressing was removed 5 to 6 days after grafting, and further dressing is not required as the graft is well adhered and sealed around the edges

* Contains calcium thioglycollate Carter Products, Inc , New York, N Y
† Winthrop Laboratories, New York, N Y

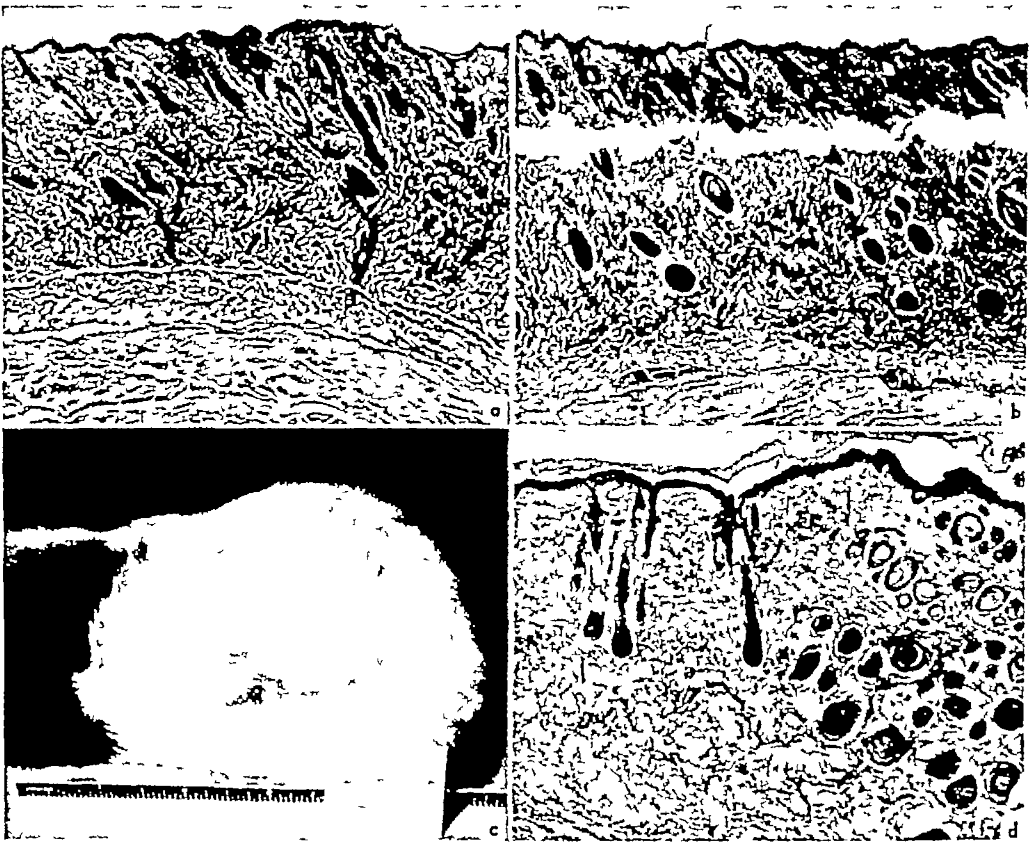


FIGURE 1 (a) Section through normal guinea pig skin, showing the depth to which skin is excised in the preparation of graft beds The dermatome setting is 45/1000 inch (1140 μ) ×18 (b) Section through normal guinea pig skin, showing depth of skin excision in the preparation of grafts of one third skin thickness The dermatome setting is 20/1000 inch (508 μ) ×18 (c) Skin isograft (4 × 6 cm) in Strain 2 guinea pig 16 weeks after grafting The graft was turned at grafting so that the direction of hair growth is opposite to that of the surrounding host skin (d) Section through the junctional area of isograft and recipient skin in a Strain 2 guinea pig 30 days after grafting Note that the normal-appearing graft (left) is distinguishable from adjoining host skin (right) by the difference in the direction of the hair follicles ×26

## *Grafting Technique*

Split-thickness skin grafts cut with a Padgett-Hood Dermatome*[6] were used as test transplants Great uniformity of grafts can be obtained with this instrument with relative ease and rapidity, as the skin can be cut at an accurately predetermined thickness and surface area In addition to the usual use of the dermatome for preparing skin grafts,[7] we also found the dermatome most effective for preparing uniform graft beds (recipient sites for grafts) of a standardized depth and size In this manner virtually complete excision of the dermis was obtained, with exposure of the panniculus carnosus and the overlying intact vascular bundles (FIGURE 1a)

* Manufactured by Kansas City Assemblage Co , Kansas City, Mo

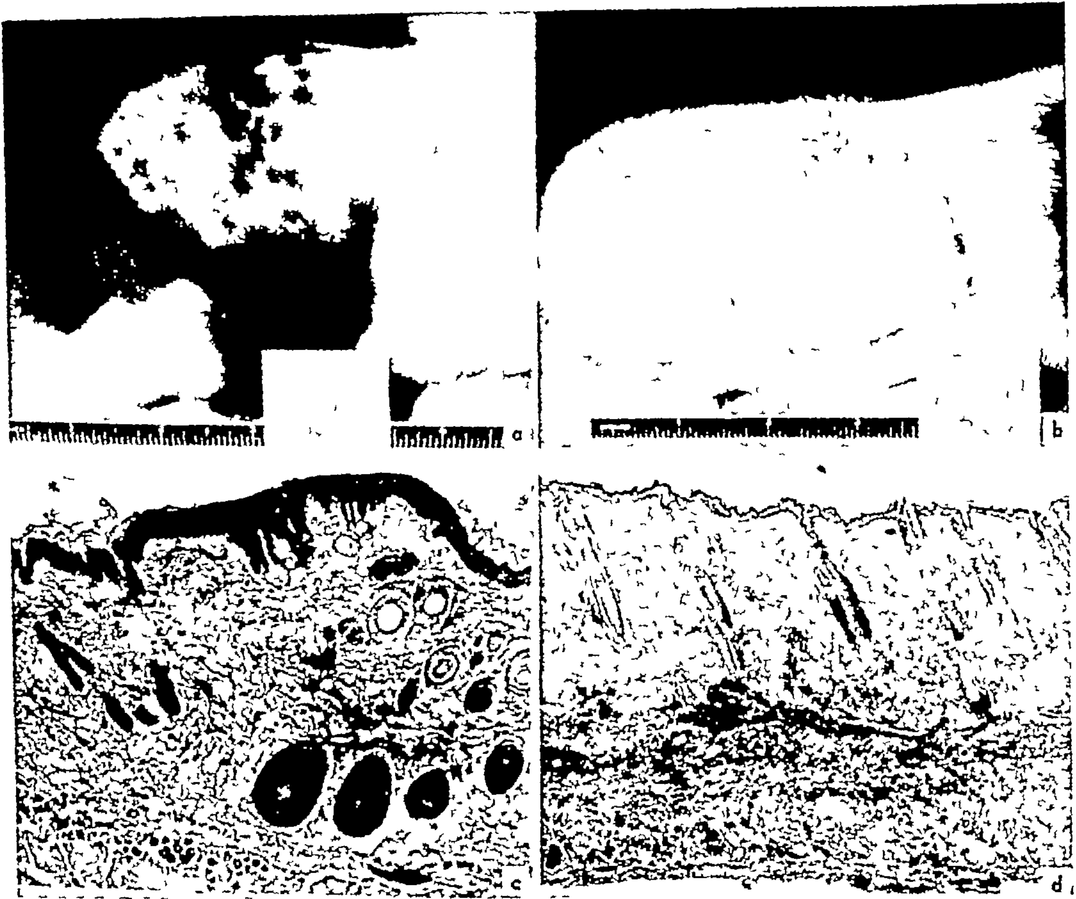



FIGURE 2 (a) Composite skin isograft in Strain 13 guinea pig with fur clipped, 16 week after grafting The left half of the graft is tan skin and the right half is white skin, both are grafted onto a patch of black host skin Note the conspicuous spread of black pigment into both grafts from the surrounding host skin (b) Skin isograft (3 × 5 cm ) in a Strain 13 guinea pig 16 weeks after grafting Note that the graft bears a full coat of hair, and that the direction of hair growth is opposite from that of the surrounding host skin (c) Section through the junction of an isograft and the host skin in a Strain 13 guinea pig 24 days after grafting Note that the difference in the direction of the hair follicles of the graft (left) and the host skin (right) is evident ×26 (d) Homograft in a Strain 2 guinea pig 2 days after grafting ×26

fore be considered together Biopsies taken at 2 days postgrafting (FIGURE 2d) show as yet unaltered donor skin incompletely healed onto its bed and held there by organizing fibrin clots At 5 days a prominent, newly formed fibrous tissue fusion of graft and bed has developed (FIGURE 3a), and the graft edges have become sealed completely by the junction of the graft and host epidermis, with sequestration of the dry, overlapping graft margins (FIGURE 3b) At this time also, marked hyperplastic proliferation of the epidermal elements and abundant mitotic cells are conspicuous Many distended vascular channels (probably lymphatics) are found throughout the graft dermis, densely packed with mononuclear lymphoid cells (FIGURE 3c) By 6 days after grafting there is a further thickening and incipient foamy appearance of the upper layers of the epidermis, and a diffuse mononuclear invasion of the graft dermis (FIGURE

TABLE 1
SKIN AUTOGRAFT AND ISOGRAFT SURVIVAL

| Type of skin graft | Donor strain | Recipient strain | No of grafts applied and surviving | Graft survival time (to date) |
|---|---|---|---|---|
| Autologous | 2 | 2 | 16 | >20 weeks |
| | 13 | 13 | 18 | >20 weeks |
| Isologous | 2 | 2 | 14 | >20 weeks |
| | 13 | 13 | 14 | >18 weeks |

## RESULTS

### *Autografts*

Following the described technique, consistently successful autograft takes were obtained, no shrinkage or scarring was observed, and the grafts are pliable and of good texture Within 2 to 4 weeks a full coat of hair was grown that displayed the color and growth direction of the donor skin and not that of the recipient site

### *Isografts (Intrastrain Homografts)*

*Strain 2* Fourteen grafts exchanged between members of Strain 2 resulted in 14 takes and graft survivals of more than 20 weeks at the time of this writing (TABLE 1) The grafts are fur-bearing, show no signs of adverse reactions or breakdown (FIGURE 1c), and are grossly as well as histologically indistinguishable from autografts Biopsies taken one month after grafting show normal skin structure, viable epidermal cells with normal staining characteristics (hematoxylin and eosin), and no leukocyte invasion Moreover, the graft is distinguishable from surrounding recipient skin only by the direction of the hair follicles (FIGURE 1d) and differences in pigmentation, if such be present Where isograft and surrounding recipient skin were of different pigmentation, conspicuous pigment spread from dark to light skin occurred (FIGURE 2a) as described in guinea pig autografts by Billingham and Medawar [8]

*Strain 13* Fourteen grafts exchanged between members of Strain 13 also resulted in uniformly successful takes and survivals to 18 weeks at the time of this writing (FIGURE 2b) As in Strain 2, the grafts are grossly and histologically indistinguishable from autografts (FIGURE 2c)

Although incompatibility ascribable to differences in sex of donor and recipient was not specifically studied, no adverse reactions were noted in grafts from male to female animals in either strain during the time span of these experiments (maximum 20 weeks)

### *Homografts (Interstrain Homografts)*

*"First Set" reaction* Fourteen grafts transplanted from Strain 13 donors onto Strain 2 recipients, as well as the reciprocal relationship of an equal number of Strain 2 grafts onto Strain 13 recipients, all ran a strikingly similar time course and pattern of graft rejection in both strains (TABLE 2) and will there-

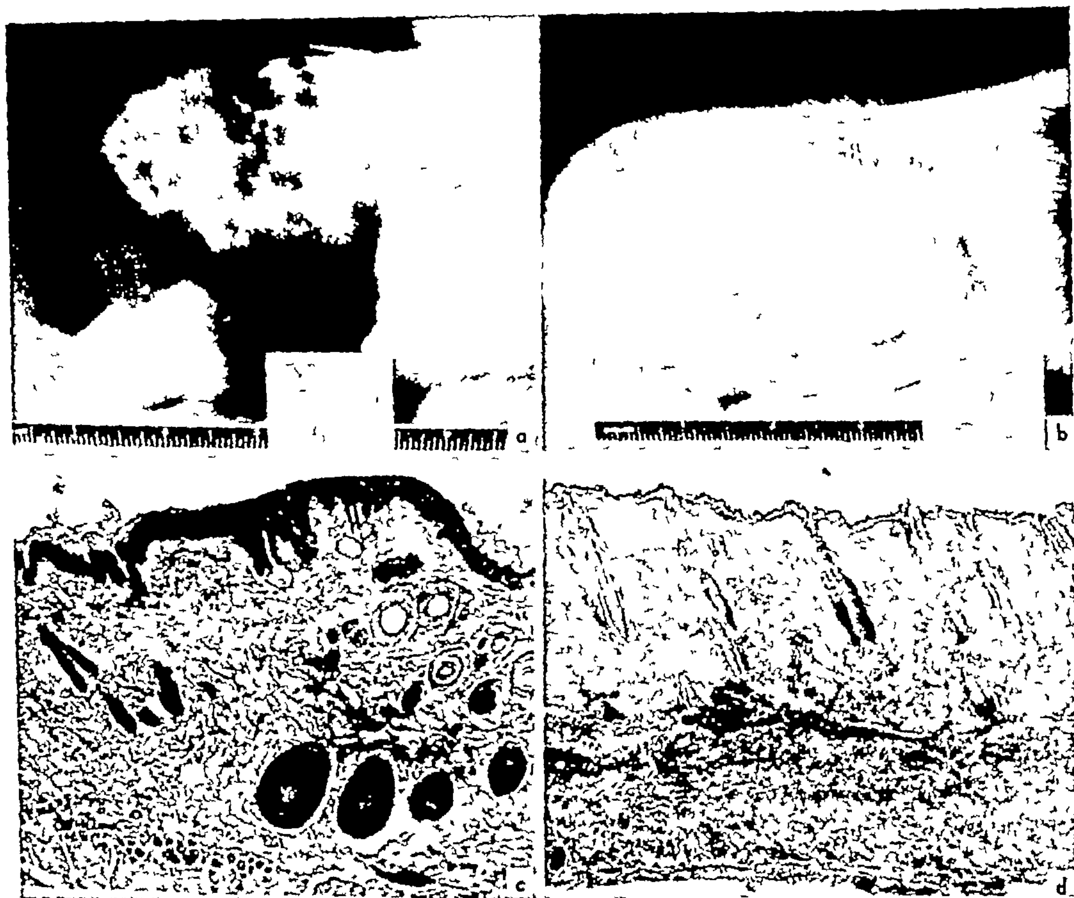


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TABLE 2
SKIN HOMOGRAFT SURVIVAL

| Type of skin graft | Donor strain | Recipient strain | No of grafts applied | No of grafts sloughed on respective postgrafting days — Days | | | | | Mean graft survival time |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 5 | 6 | 7 | 8 | 9 | |
| Homologous | | | | | | | | | |
| "First Set" | 13 | 2 | 14 | | | 5 | 8 | 1 | 7 7 (±0 3) days |
| | 2 | 13 | 14 | | | 4 | 9 | 1 | 7 8 (±0 3) days |
| "Second Set" | 13 | 2 | 10 | * | | | | | 0 |
| | 2 | 13 | 6 | * | | | | | 0 |

* No primary takes, "white" avascular grafts at first inspection 5 days postgrafting

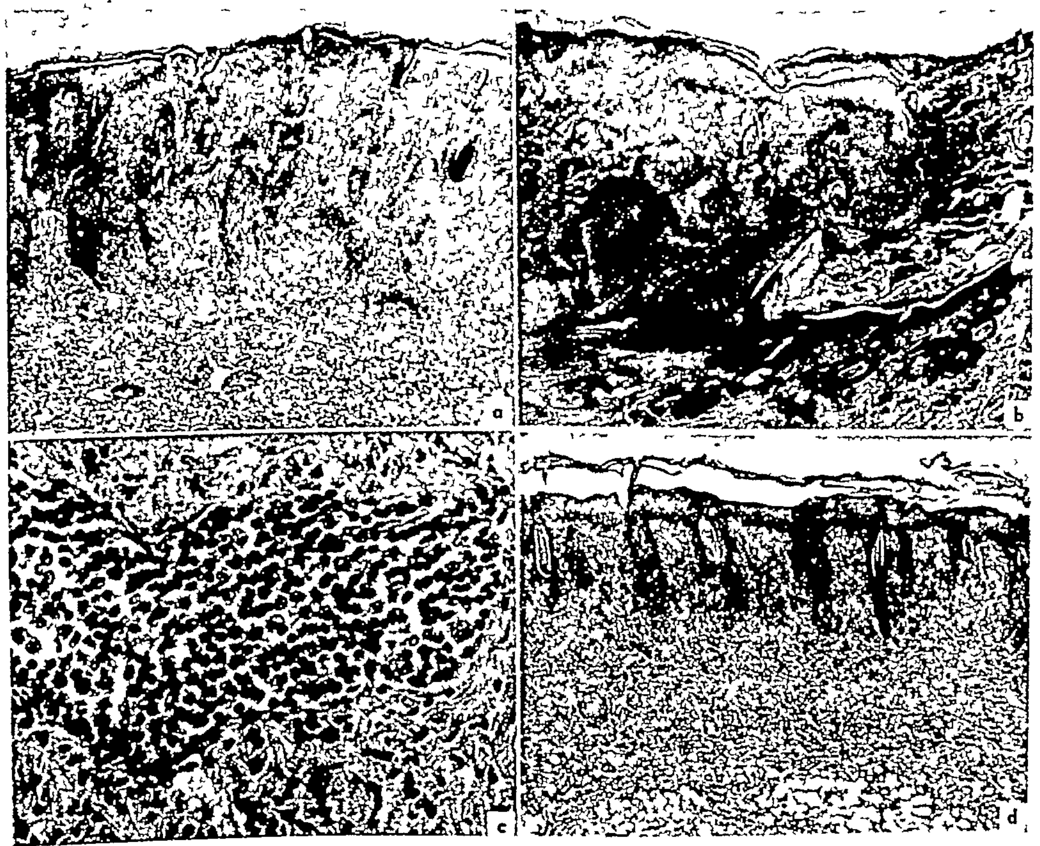



FIGURE 3 (a) Homograft in a Strain 2 guinea pig 5 days after grafting ×26 (b) Homograft of a Strain 2 guinea pig 5 days after grafting, showing junction of graft (left) and host skin ×26 (c) Lymphatic vessels packed with mononuclear lymphoid cells in a homograft of a Strain 2 guinea pig 5 days after grafting ×180 (d) Homograft in a Strain 13 guinea pig 6 days after grafting ×20

3d). At no time during the graft destruction was a preponderantly polymorphonuclear invasion observed Seven days postoperatively the bright pink color of the graft, prevalent from the second to the fifth or sixth day, begins to darken to deep red, occasional petechial hemorrhages appear and, on the eighth day, a frankly hemorrhagic, dark brown, crusted appearance sets in Beginning about the fifth day the progressive induration and swelling lead to a very striking thickening of the entire graft (as much as tenfold), and the graft is conspicuously raised above the level of the surrounding host skin (FIGURE 4a) Microscopically, 7-day grafts reveal patchy breakdown of the germinative layer, with foamy cytoplasm and nuclear pyknosis, marked thickening and spongy hyalinization of the entire epidermis, loss of structural detail of the collagen fibers of the dermis, and abundant invasion by mononuclear leukocytes (FIGURE 4b) A most characteristic feature is the massive displacement of graft dermal elements by the fingerlike downgrowth of proliferative epidermal projections By the eighth day, structural detail is diffusely obscured, and the epidermal germinative layer has completely disintegrated except for some viable-appearing

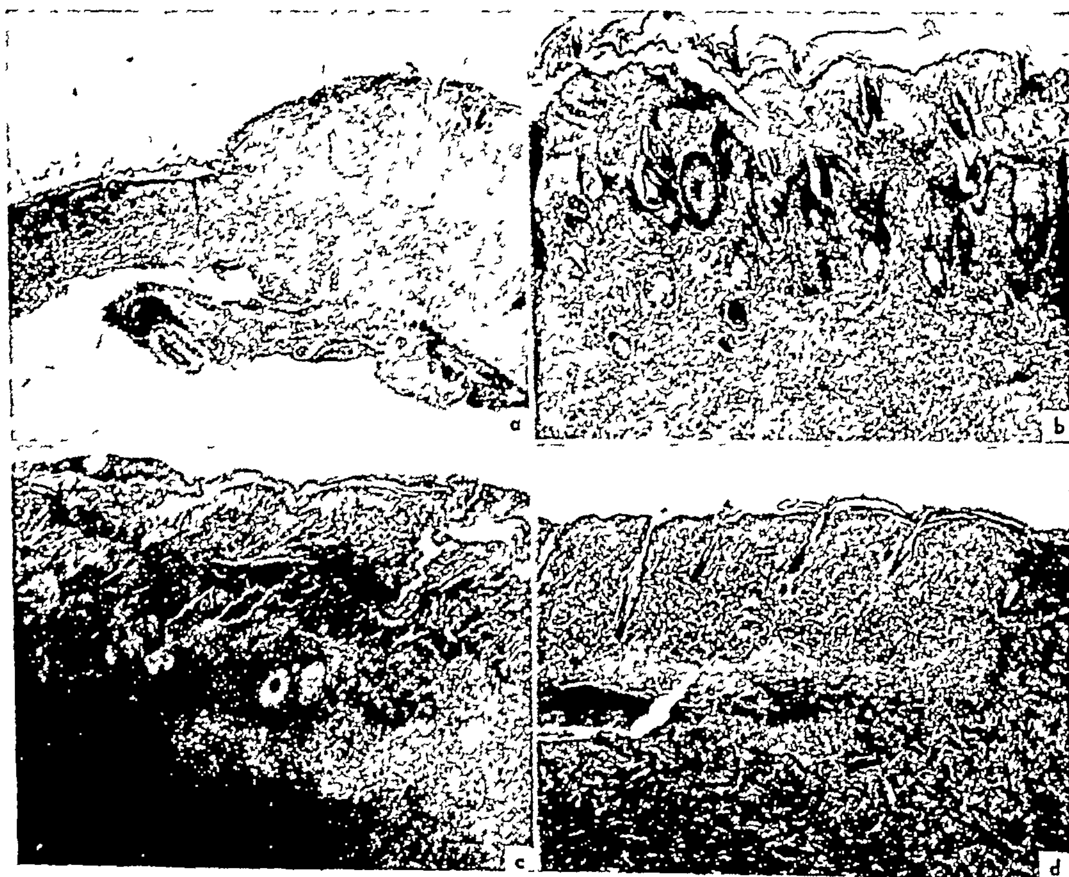



FIGURE 4 (a) Section through a markedly thickened homograft (right) and the adjoining normal host skin of a Strain 13 guinea pig 7 days after grafting ×5 8 (b) Homograft in a Strain 13 guinea pig 7 days after grafting ×20 (c) Homograft in a Strain 13 guinea pig 8 days after grafting ×20 (d) "Second-set" homograft in a Strain 2 guinea pig 7 days after grafting Note the necrotic, avascular graft and the "black line" that marks the contact, without fusion, of the graft and its bed ×22

TABLE 2
SKIN HOMOGRAFT SURVIVAL

| Type of skin graft | Donor strain | Recipient strain | No of grafts applied | No of grafts sloughed on respective postgrafting days | | | | | Mean graft survival time |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Days | | | | | |
| | | | | 5 | 6 | 7 | 8 | 9 | |
| Homologous | | | | | | | | | |
| "First Set" | 13 | 2 | 14 | | | 5 | 8 | 1 | 7 7 (±0 3) days |
| | 2 | 13 | 14 | | | 4 | 9 | 1 | 7 8 (±0 3) days |
| "Second Set" | 13 | 2 | 10 | * | | | | | 0 |
| | 2 | 13 | 6 | * | | | | | 0 |

* No primary takes, "white" avascular grafts at first inspection 5 days postgrafting

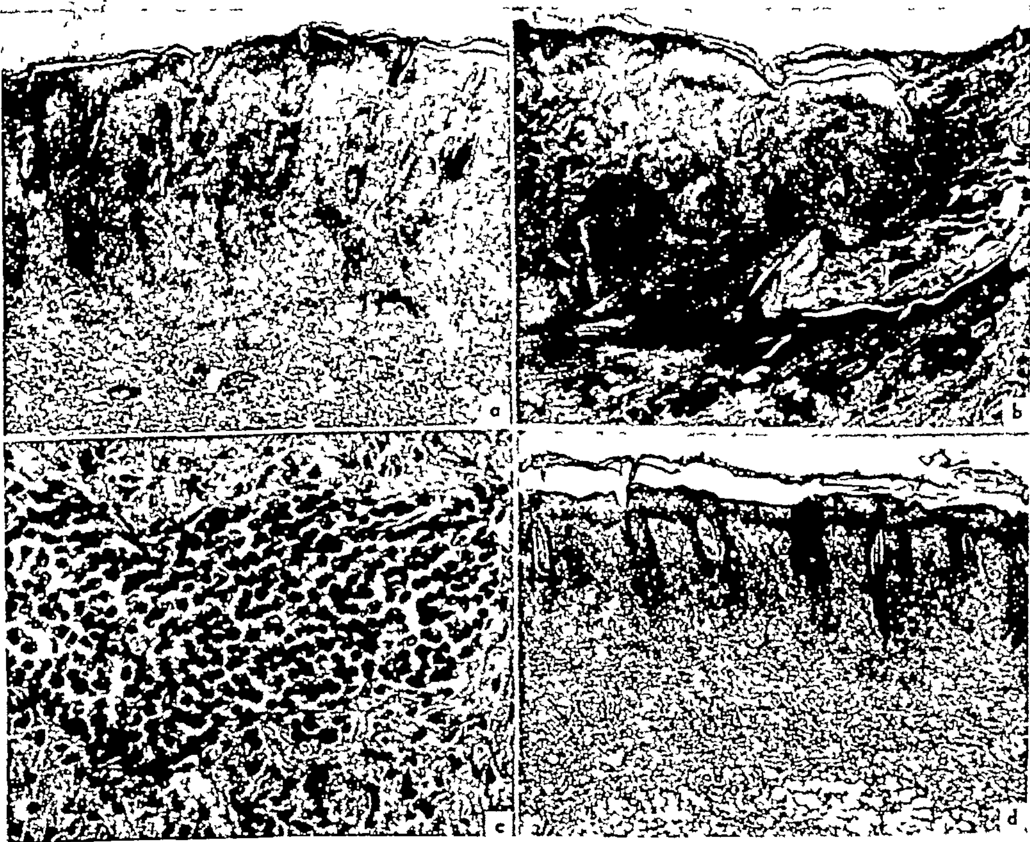


FIGURE 3 (a) Homograft in a Strain 2 guinea pig 5 days after grafting ×26 (b) Homograft of a Strain 2 guinea pig 5 days after grafting, showing junction of graft (left) and host skin ×26 (c) Lymphatic vessels packed with mononuclear lymphoid cells in a homograft of a Strain 2 guinea pig 5 days after grafting ×180 (d) Homograft in a Strain 13 guinea pig 6 days after grafting ×20

3d). At no time during the graft destruction was a preponderantly polymorphonuclear invasion observed Seven days postoperatively the bright pink color of the graft, prevalent from the second to the fifth or sixth day, begins to darken to deep red, occasional petechial hemorrhages appear and, on the eighth day, a frankly hemorrhagic, dark brown, crusted appearance sets in Beginning about the fifth day the progressive induration and swelling lead to a very striking thickening of the entire graft (as much as tenfold), and the graft is conspicuously raised above the level of the surrounding host skin (FIGURE 4a) Microscopically, 7-day grafts reveal patchy breakdown of the germinative layer, with foamy cytoplasm and nuclear pyknosis, marked thickening and spongy hyalinization of the entire epidermis, loss of structural detail of the collagen fibers of the dermis, and abundant invasion by mononuclear leukocytes (FIGURE 4b) A most characteristic feature is the massive displacement of graft dermal elements by the fingerlike downgrowth of proliferative epidermal projections By the eighth day, structural detail is diffusely obscured, and the epidermal germinative layer has completely disintegrated except for some viable-appearing

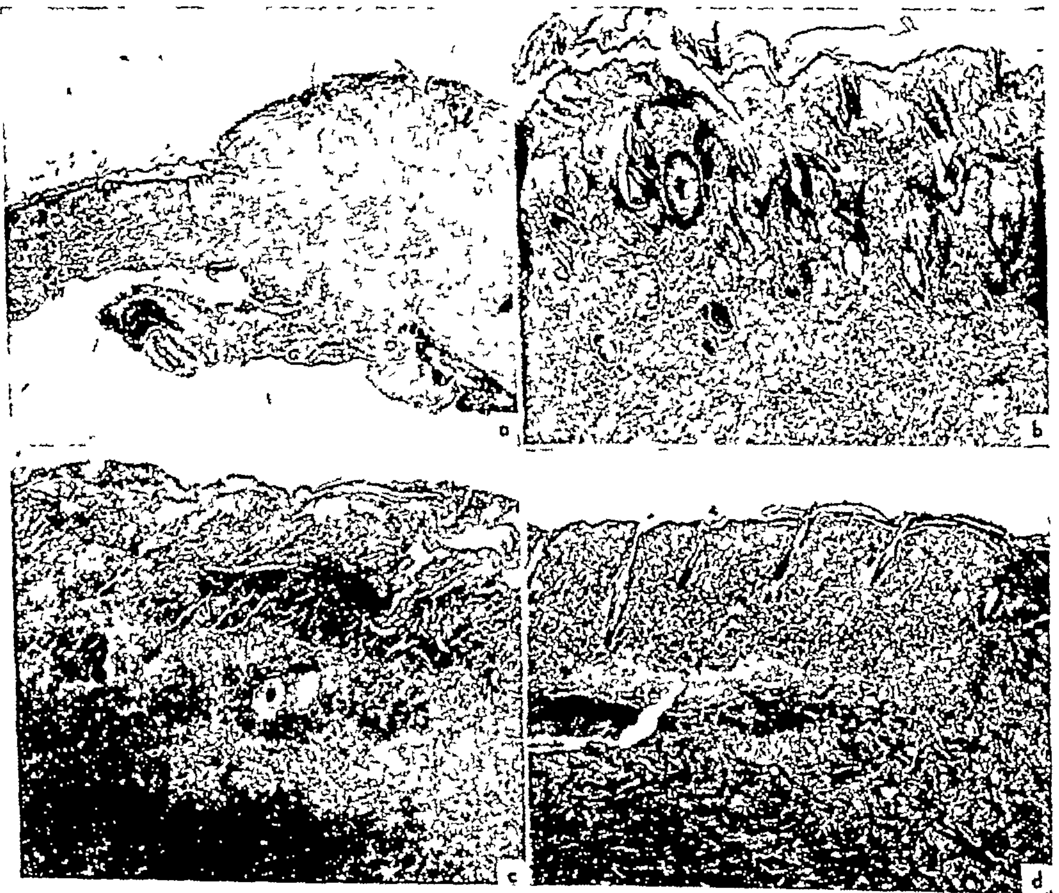



FIGURE 4 (a) Section through a markedly thickened homograft (right) and the adjoining normal host skin of a Strain 13 guinea pig 7 days after grafting ×5 8 (b) Homograft in a Strain 13 guinea pig 7 days after grafting ×20 (c) Homograft in a Strain 13 guinea pig 8 days after grafting ×20 (d) "Second-set" homograft in a Strain 2 guinea pig 7 days after grafting Note the necrotic, avascular graft and the "black line" that marks the contact, without fusion, of the graft and its bed ×22

deep perifollicular cells, and subepithelial hemorrhages are frequent (FIGURE 4c). The complete separation of the crusted, friable, weeping graft follows during the next day or two. Mean homograft survival times, judged by the end point of histological necrosis of the major extent of the epithelial germinative layer, were 7.7 (±0.33) days and 7.8 (±0.31) days for Strain 2 and Strain 13 recipients, respectively; no graft persisted longer than 9 days.

*"Second-set" reaction.* Ten Strain 2 animals, having previously rejected Strain 13 homografts, were grafted a second time, 10 to 12 weeks after their first experience with Strain 13 skin grafts, onto the opposite thoracoabdominal area. On removing the dressing 5 days after grafting every graft was found to be pale white and totally avascular. Necrotic decomposition was noted, with epithelial dehiscence but no edema or induration. The grafts were undermined by blebs of clear serous exudate; complete nonunion of graft and bed allowed easy dislodgment of the dead grafts. The graft beds were lined by abundant dark red granulation tissue and there was no evidence of gross infection. Histologically, at 5 and 7 days after grafting a totally devitalized graft devoid of viable appearing epithelial cells was seen lying on its bed with areas of dehiscence therefrom. A prominent basophilic line ("black line") composed of a dense network of mononuclear spindle-shaped cells (fibroblasts?) marked the line of contact (but not fusion) of graft and bed (FIGURE 4d). Similar cells in large numbers filled the interstices of the host dermal collagen fibers, and erythrocyte extravasations were frequent. Except for occasional pyknotic nuclei, the graft stained diffusely eosinophilic (hematoxylin and eosin); fragmentation of the graft collagen fibers was evident, but no host cell invasion had occurred. No evidence of graft vascularization or primary healing into place was detected.

Six Strain 13 animals, having rejected Strain 2 homografts 10 weeks previously, similarly showed a complete lack of primary healing and nonvascularization of the second homograft. Again, none of the grafts manifested any of the characteristic color changes, induration, or swelling described for first-set homografts. As a control measure, these same 6 Strain 13 animals, having shown the "white" graft second-set reaction, were isografted with skin from Strain 13 donors 10 days later and responded with primary takes and survival of every isograft not unlike those in animals never previously grafted.

## Discussion

The uniformly rapid time course and violent rejection reaction of first-set grafts observed in these studies implies a high sensitivity to this type of immunological stimulation, an impression that gains further support from the response pattern to second-set grafts. The avascular white graft second-set reaction is interpreted as a manifestation of a high level of tissue immunity and is a graft rejection phenomenon qualitatively distinct from a first graft rejection, as well as quite unlike the usual findings in other animals (for example, in the mouse) under the same conditions. The induction of levels of hypersensitivity sufficiently high to result in white graft reactions apparently requires, in other animal species, repeated or prolonged and much more powerful anti-

genic stimulation, such as results from incorporating the antigen (for example, spleen cells) into *Mycobacterium*-paraffin oil adjuvants, as described by Stetson and Demopoulos elsewhere in this monograph. This striking dissimilarity between first- and second-set reactions (if confirmed with the same uniformity in larger numbers of animals) should prove these guinea pigs to be most suited for identification of the "homograft antigens" involved by testing putatively antigenic cell fractions.[9] These potential applications emerge from the clear distinction between the two reaction patterns, and also from the apparently high degree of reactivity these animals show to homograft antigen stimulation. The marked sensitivity-reactivity response to this type of antigenic stimulation might reasonably be expected to affect the ease of induction of "actively acquired tolerance," as well as the completeness of the resultant tolerance and its refractoriness to subsequent test grafting. Although presently it is possible only to speculate about the ease of induction of homograft tolerance in guinea pigs (relative to other animal species such as mice[10] and rats[11]), an attempt to induce "specific inhibition" of tuberculin sensitization in guinea pigs resulted in partial inhibition ("tolerance") only, reversible by BCG challenge of the adult animals.[12]

The guinea pig, presently the largest animal available of adequate inbreeding to display histocompatibility, invites the possibility of confirming, by transplantation of major organs, some of the findings so far almost exclusively based on skin grafts. However, aside from these considerations, the guinea pig offers additional and possibly unique advantages in tissue transplantation studies, two of which are of special interest. First, as the homograft rejection is generally recognized to be an immune reaction of the delayed, tuberculin type, the guinea pig, as the experimental animal of choice for the study of tuberculin type immune reactions, provides an excellent opportunity for the comparative investigation of both of these phenomena in the same individual as well as in the same species. Second, all known cytotoxic reactions require the presence of serum complement. Since the reactions of guinea pig complement are by far the most thoroughly studied and understood, the opportunity arises to elucidate the exact role of complement and its components in the homograft rejection reaction. In view of the recent weighty implication of serum complement as one essential factor in eliciting the tissue injury associated with an *in vivo* immune reaction of the immediate type by Osler *et al.*,[13] it would appear most profitable to investigate the participation of complement in *in vivo* immune tissue injuries of the delayed type, more specifically, in homotransplant injury.

## Summary

(1) The application of the Padgett-Hood Dermatome to split-thickness skin transplantation in guinea pigs is presented. This technique yields both grafts and graft beds of uniform, accurately predetermined dimensions.

(2) Data are presented to show complete intrastrain histocompatibility within each of 2 strains of inbred guinea pigs.

(3) First-set interstrain skin homografts between the 2 strains of inbred guinea pigs undergo a violent graft rejection, culminating uniformly on the eighth day after grafting These rejections are described sequentially

(4) Second-set interstrain skin homografts result in characteristic nonvascularized white grafts that are qualitatively distinct from first-set rejection

(5) The implications of the observed histocompatibility, the high homograft reactivity and other characteristics peculiar to guinea pigs that are advantageous in transplantation studies are discussed

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## Discussion

1 Sparrow, E M 1953. The behaviour of skin autografts and skin homografts in the guinea pig with special reference to the effect of cortisone acetate and ascorbic acid on the homograft reaction J Endocrinol **9** 101
2 Woodruff, M F A 1954 The effect of cortisone and thyrotrophin on thyroid transplants in the guinea pig J Endocrinol **11** 1
3 Billingham, R E, L E Brent & N A Mitchison 1957 The route of immunization in transplantation immunity Brit J Exptl Pathol **38**· 467
4 Wright, S 1922 The effect of inbreeding and cross-breeding on guinea pigs U S Dept Agr Bull **No 1090**
5 Loeb, L 1945 The Biological Basis of Individuality Chap 8 Thomas Springfield, Ill
6 Padgett, E C 1939 Calibrated intermediate skin grafts Surg Gynecol Obstet **69.** 779
7 Woodruff, M F A & L O Simpson 1955 Experimental skin grafting in rats Plastic Reconstr Surg **15** 451
8 Billingham, R E & P B Medawar 1948 Pigment spread and cell heredity in guinea pig skin Heredity **2** 29
9 Billingham, R E, L Brent & P B Medawar 1956 Antigenic stimulus in transplantation immunity Nature **178** 514
10 Billingham, R E & L Brent 1957 A simple method for inducing tolerance of skin homografts in mice Transplantation Bull **4**· 67
11 Woodruff, M F A 1957 Postpartum induction of tolerance to homologous skin in rats Ann N Y Acad Sci **64**(5) 792
12 Weiss, D W & A Q Wells 1957 Actively acquired tolerance to tuberculoprotein Nature **179.** 968
13 Osler, A G, M M Hawrisiak, Z Ovary, M Siqueira & O G Bier 1957 Studies on the mechanism of hypersensitivity phenomena II The participation of complement in passive cutaneous anaphylaxis of the albino rat J Exptl Med **106** 811

# LYMPHATIC REPAIR AND THE GENESIS OF HOMOGRAFT IMMUNITY

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If it is accepted that the rejection of tissue homografts is an immune response on the part of the host, a satisfactory anatomical analysis of the phenomenon must answer the following questions, among others (1) What is the nature of the antigens involved and what is their specific cellular locus? (2) Which cells of the host are involved in the genesis of the immune response? (3) How do these cells (or their products) reach the graft to effect the immune reaction?

Insofar as skin homografts are concerned, partial answers to these questions are already available As for the first, the work of Billingham *et al* , 1956, indicates that the antigens are probably within the nuclei of epidermal cells As for the second, the response of the host to an orthotopic skin homograft occurs principally in the first regional lymph node draining the graft and manifests itself in the appearance of "large lymphoid cells" (see Scothorne, 1957) As for the third, the graft will survive when the cells of the graft are protected from contact with host cells as, for example, in a naturally avascular tissue such as cornea and cartilage or, fortuitously, in nonvascularized grafts in the anterior chamber of the eye or when the graft is insulated by a millipore membrane (Algire *et al* , 1957, Woodruff, 1957) It seems likely, therefore, that host *cells* are responsible for the destruction of the graft and that they reach it by way of the blood stream

A fourth problem, namely, how the antigens reach the antibody-forming apparatus of the host, has been less intensively studied than the other three It is clear that they are transported to the regional lymph node by host lymphatics rather than by the blood stream (Scothorne, 1957), but it is not certainly known if graft antigens enter the host lymphatics in the graft bed directly or if they must await the repair of the lymphatic pathways within the graft

The question is of both special and general interest If the antigens of a skin homograft are sequestered until the lymphatic vessels within the graft are repaired, experimental interference with lymphatic repair might prolong the survival of the graft by reducing its effective antigenicity More generally, the prolonged survival of tissues naturally lacking in intrinsic lymphatics (cornea and cartilage) or experimentally denied them (tissues in millipore chambers) might be due as much to the resulting low effective antigenicity as to any of the customary explanations

Earlier studies of lymphatic repair in grafted skin in rabbits have given conflicting results Cavalli, 1935, using injected specimens, reported that the lymphatic drainage of skin autografts is fully restored by the fourth or fifth day after grafting McGregor and Conway, 1956, using a different technique, found that in autografts of skin the restoration of lymphatic drainage was usually delayed until the sixth day, while skin homografts underwent destruc-

TABLE 1

INJECTED LYMPHATICS IN GRAFT

| | Autograft | Homograft |
|---|---|---|
| Day 3 | – | – – – – |
| Day 3½ | – | – – – – – |
| Day 4 | – – – – | – – – |
| Day 5 | + + + + + + + + + – – | + + + + + |
| Day 6 | + + + + | + + |
| Day 7 | + + + + | |
| Day 9 | + + + | |
| Day 13 | + + | |

Each symbol represents a single graft. – = no injection of graft lymphatics, + = injection of graft lymphatics.

tion without ever showing the re-establishment of lymphatic drainage. Egdahl and Varco, 1956, on the other hand, found that intradermally injected fluorescein was removed as rapidly and fully from a fifth-day homograft as from normal skin, and they interpret this as indicating full restoration of lymphatic drainage by that time.

In the present experiments guinea pigs have been used. Full-thickness autografts or homografts were made to the ears and, at intervals after grafting, the animals were sacrificed and the lymphatics of the ear injected with India ink. Since the injections were made into the lymphatics of the host, lymphatics within the graft were filled by retrograde flow, and filling of the graft lymphatics provided unequivocal evidence of continuity between graft and host lymphatics. TABLE 1 summarizes the results obtained.

The following conclusions may be drawn from these results.

(1) The technique is a reliable one. Among 15 cases on the sixth and subsequent days there was no failure to inject graft lymphatics.

(2) There is no lymphatic repair by the fourth day. Universal negatives are always difficult to establish, but it seems unlikely, to say the least, that a technique that is almost invariably successful on the fifth and following days should be invariably unsuccessful before the fifth day.

(3) Lymphatic repair occurs at the same time in autografts and homografts.

(4) The restoration of lymphatic drainage of skin grafts occurs by anastomosis of host vessels lying in the graft bed with surviving intrinsic vessels within the graft. The evidence for this view is as follows: (a) up to 4 days no lymphatics were injected in the graft but, at the fifth day, the graft lymphatics were in many cases fully filled. One would not get a clear-cut demarcation like this if regenerating host lymphatics were gradually penetrating the graft from the graft bed, (b) the pattern of injected lymphatics was fully mature, that is, the same as in normal skin, at the fifth day, and did not change on subsequent days.

All the injections were carried out under direct visual control, using the binocular microscope. In nearly all cases filling of the graft lymphatics took place by retrograde flow of ink from deep vessels of the graft bed. Rarely was

there any continuity between the superficial subepidermal plexuses of host and graft

Having established the normal anatomical pattern of lymphatic repair, the next problem was to relate the time of repair to the onset of homograft destruction Here the results, though very suggestive, are not absolutely conclusive. As in the lymphatic injection experiment, homografts were made to the ears of a stock brown and white strain, using albino animals as donors The length of survival was very variable and, in some cases, the epidermis was still alive, although extensively infiltrated by lymphocytes, on the fifteenth day after grafting With such extended survival one cannot exclude the possibility that immunization of the host did not begin until after the fifth day, when lymphatic repair is complete The interval between 5 and 15 days would seem to allow ample time for the development and expression of the immune response

Less equivocal results are given by those animals in which graft breakdown occurred earliest Histologically, 3 eighth-day grafts showed breakdown of long standing, while 3 at the seventh day and 1 at the sixth day showed extensive lymphocytic infiltration of the epidermis In view of the earlier results it may be assumed that in these animals the intrinsic lymphatic drainage of the graft was not restored until the fifth day It seems unlikely that the interval between this event and the early signs of graft breakdown was long enough for the development and expression of the immune response A more reasonable conclusion would be that immunization of the host had already begun before the fifth day, that is, before restoration of the intrinsic lymphatic drainage of the graft

Although not finally proved, it seems likely that the antigens of the graft reach the antibody-forming apparatus of host directly via the host lymphatics in the graft bed, and that immunization of the host can occur in the absence of an intrinsic lymphatic drainage of the graft A similar conclusion was reached by McGregor and Conway, 1956, using a different species and a different method

*Summary* In guinea pigs the lymphatic drainage of homografts and autografts of ear skin is restored on the fifth day after grafting by the anastomosis of host lymphatics in the graft bed with pre-existing intrinsic lymphatics of the graft

*References*

ALGIRE, G H, J M WEAVER & R T PREHN 1957 Studies on tissue homotransplantation in mice using diffusion-chamber methods Ann N Y Acad Sci 64(5): 1009–1013

BILLINGHAM, R E, L BRENT & P B MEDAWAR 1956 The antigenic stimulus in transplantation immunity Nature **178.** 514–519

CAVALLI, M 1935 Sul comportamento dei linfatici nell innesto autoplastico della pelle Sperimentale **89.** 504–508

EGDAHL, R H & R L VARCO 1956 Intradermal fluorescein test for homograft rejection period Transplantation Bull **3** 152–153

MCGREGOR, I A & H CONWAY 1956 Development of lymph flow from autografts and homografts of skin Transplantation Bull **3.** 46–47

SCOTHORNE, R J 1957 Studies on the response of the regional lymph node to skin homografts Ann N Y Acad Sci 64(5)· 1028–1038

WOODRUFF, M F A 1957 Cellular and humoral factors in the immunity to skin homografts experiments with a porous membrane Ann N Y Acad Sci 64(5): 1014–1026

# STUDIES OF TRANSPLANATION IMMUNITY IN HAMSTERS*

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## *Introduction*

Unlike all other common laboratory mammals, Syrian hamsters (*Mesocricetus auratus*) appear to be unique in that they will often accept homografts of malignant tissues and, on occasion, even heterografts of both normal and malignant tissues (Toolan, 1955, Foley and Handler, 1957, Pierce, Verney, and Dixon, 1957) A detailed study has therefore been undertaken to determine whether the Syrian hamster's response to skin homografts also differs from that of other animals The adult Syrian hamsters employed were obtained from four different closed colonies in Britain, each of these was known to have been completely isolated for an extended period, although none of them had been deliberately inbred by successive brother-to-sister matings (TABLE 1) Male animals were used for most of this work

In all of the experiments to be described, two full-thickness skin grafts about 7 or 8 mm in diameter, from which all adherent fascial tissue had been removed, were transplanted to beds of the appropriate size prepared in the skin of the chest of the recipient Primary inspection of the grafts was carried out on the eighth postoperative day, and subsequent inspections were made at 2- or 3-day intervals for the first 3 weeks and less frequently thereafter Animals whose grafts survived were kept under observation for at least 100 days The survival times of the homografts were assessed on the basis of their outward appearance (Billingham and Medawar, 1951), and confirmed as required by the histological examination of biopsy specimens

*The fate of intracolony homografts* The majority of the homografts exchanged between hamsters derived from the same colony were accepted by their hosts and regenerated perfectly normal hair crops just as if they were autografts These grafts remained in perfect condition until the experiments were terminated after 100 days or longer The numbers of animals that fully accepted their intracolony homografts are given in FIGURE 1 However, the grafts on some of the animals did become inflamed and edematous and were infiltrated by lymphocytes, their complete destruction followed within a few days of the onset of these typical symptoms of incompatibility The breakdown of the grafts on all except one of the animals that rejected its homografts was complete within 3 weeks In the exceptional animal of the CB colony whose grafts survived for about 35 days, and in other recipients in later experiments to be described below, the onset of the reaction was delayed and was of the mild, chronic type There was a prolonged weak inflammation of

* The work described in this paper was done at the Department of Zoology, University College, London, England, and was supported by a grant from the British Empire Cancer Campaign

TABLE 1
COLONIES OF HAMSTERS EMPLOYED

| Colony designation | Phenotype | No years closed |
|---|---|---|
| MHA | Albino | 3 |
| MH | Agouti | 26 |
| LSH | Agouti | 5 |
| CB | Agouti | 11 |

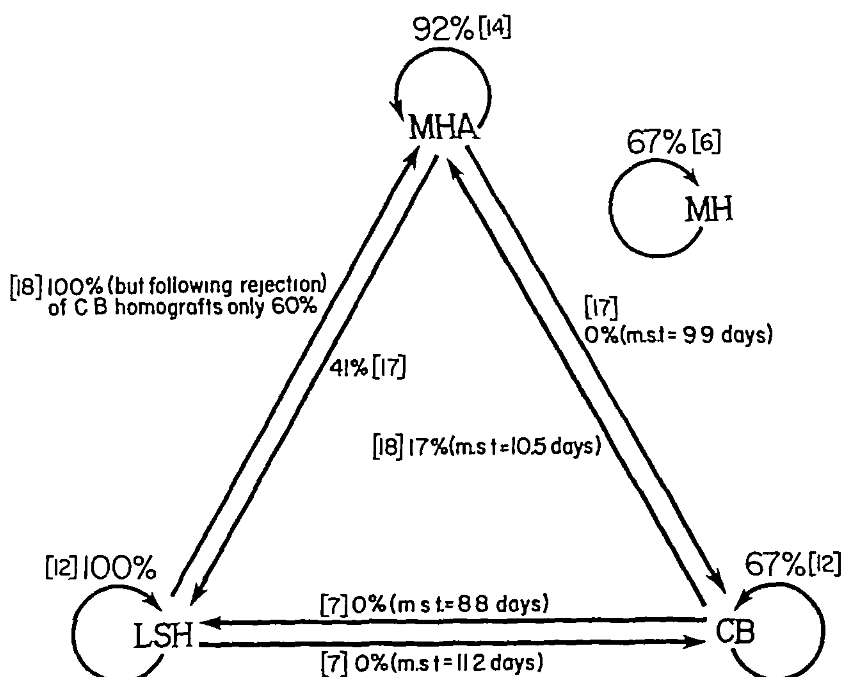



FIGURE 1 Summary of fates of skin homografts exchanged between 4 different closed colonies of Syrian hamsters, MHA, MH, CB, and LSH, in different donor-recipient combinations The closed circuits indicate intracolony homografts, all the straight arrows indicate intercolony homografts In all cases the percentage of the homografts that lived for at least 100 days is given, with the number in parentheses indicating the number of animals tested In cases where most or all of the grafts were rejected, the median survival time (m s t ) is given also

the graft dermis, progressive loss of hair, and weakening of the attachment of the superficial epidermis, accompanied by contracture of the graft as a whole The end points of these chronic homograft reactions could not be assessed with much precision

*The fate of intercolony homografts* Since the high proportion of surviving homografts might have resulted from inbreeding in the various colonies tested, skin homografts were reciprocally exchanged between hamsters derived from *different* colonies, as illustrated in FIGURE 1, in an attempt to broaden the genetic disparity, and therefore the antigenic disparity, between donors and hosts Using hamsters from 3 colonies, all of the 6 possible intercolony combinations were tested

With the combination of LSH → MHA every recipient accepted its homografts for well over 100 days without the slightest indication of a reaction It is of some interest to note that this group included 3 females that failed to react against grafts from male donors, since it has been established with all inbred

strains of mice thus far investigated that isologous skin grafts from male donors do not survive permanently if they are transplanted to female recipients, although isologous female grafts are normally acceptable to males (Eichwald *et al*, 1957) Successful intercolony homografts are illustrated in FIGURES 2 and 3

The existence of isoantigenic differences reflected in graft rejections became evident from an early stage with the reciprocal combination, MHA → LSH Only 7 of 17 of the recipients still bore surviving homografts by day 100, and weak chronic reactions were then in progress in the grafts on 2 of these animals In 5 of the 10 animals that rejected their grafts, breakdown was complete within 14 days These results are indicative of antigenic and, therefore, genetic, diversity in one or both of the MHA and LSH colonies that was not apparent from the intracolony grafting tests (FIGURE 1)

With the combination MHA → CB all animals destroyed their homografts, 16 of them within 22 days The median survival time was 9 9 (8 5 to 11 6) days The grafts on the exceptional animal survived until day 27 after a rather prolonged reaction The fact that this animal was pregnant at the time of operation may have been responsible for extending the life of its homografts Heslop, Krohn, and Sparrow (1954) have shown that pregnancy in the rabbit may prolong the life of homografts, probably as a consequence of increased corticosteroid secretion, since the administration of cortisone is known to prolong the survival of homografts in this species (Billingham, Krohn, and Medawar, 1951)

With the CB → MHA combination 13 of the 18 recipients destroyed their homografts within 2 weeks, but 3 of the remainder had failed to reject their grafts after 100 days Nevertheless, a median survival time of 10 5 days with confidence limits 9 5 to 11 5 days for 19 20 probability was estimated for this combination (Litchfield, 1949)

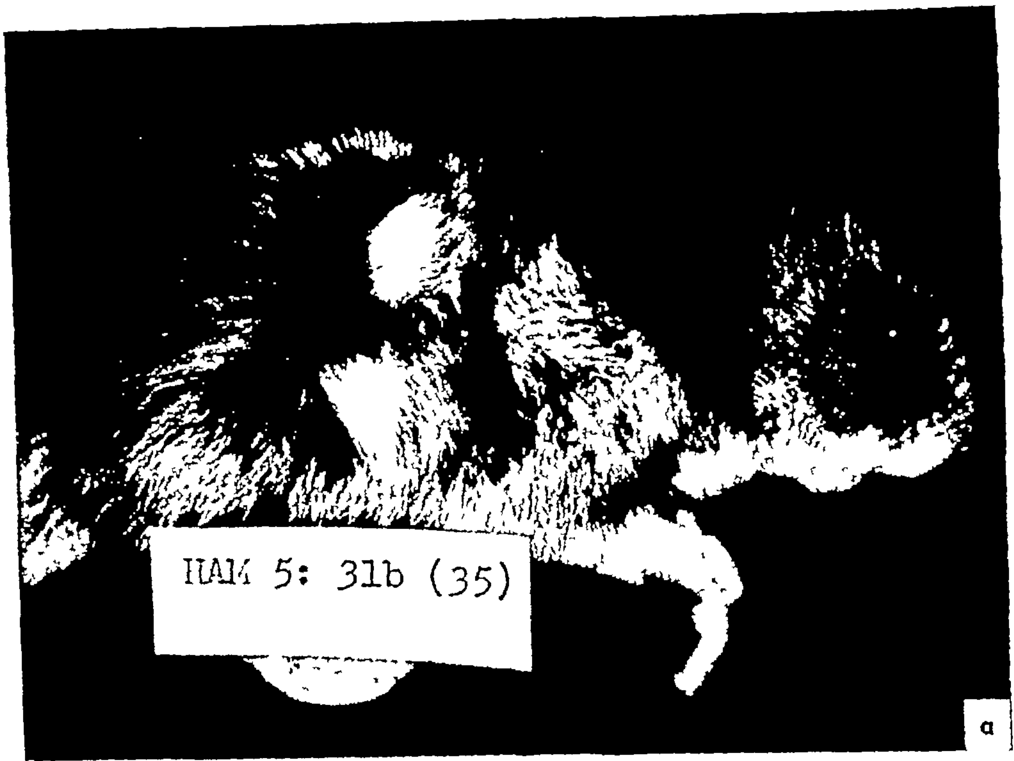
Finally, with the colony combination LSH → CB and vice versa, all animals dismissed their homografts within 3 weeks, the median survival times being 11 2 (8 9 to 14 1) days and 8 8 (7 6 to 10 2) days, respectively

Nine of the recipients from the combinations MHA → CB and CB → MHA that had rejected their homografts were again grafted with skin from their original donors 3 weeks after the first operation These second-set grafts healed in very weakly and underwent the typical accelerated rejection characteristic of homografts transplanted to previously sensitized recipients in other species These various findings show that Syrian hamsters given the proper stimulus are just as capable of reacting against homografts as other mammals

*Specificity tests* The specificity of a Syrian hamster's capacity to accept or reject skin homografts was investigated by 3 different experiments

(1) Six LSH animals that had promptly rejected grafts from MHA donors were each given subsequent homografts from one or more *different* MHA donors Three of these animals fully accepted the homografts from at least one new MHA donor for the duration of the experiment

(2) Eleven of the 13 MHA hamsters that had destroyed homografts from CB donors within 14 days were given subsequent homografts from LSH donors



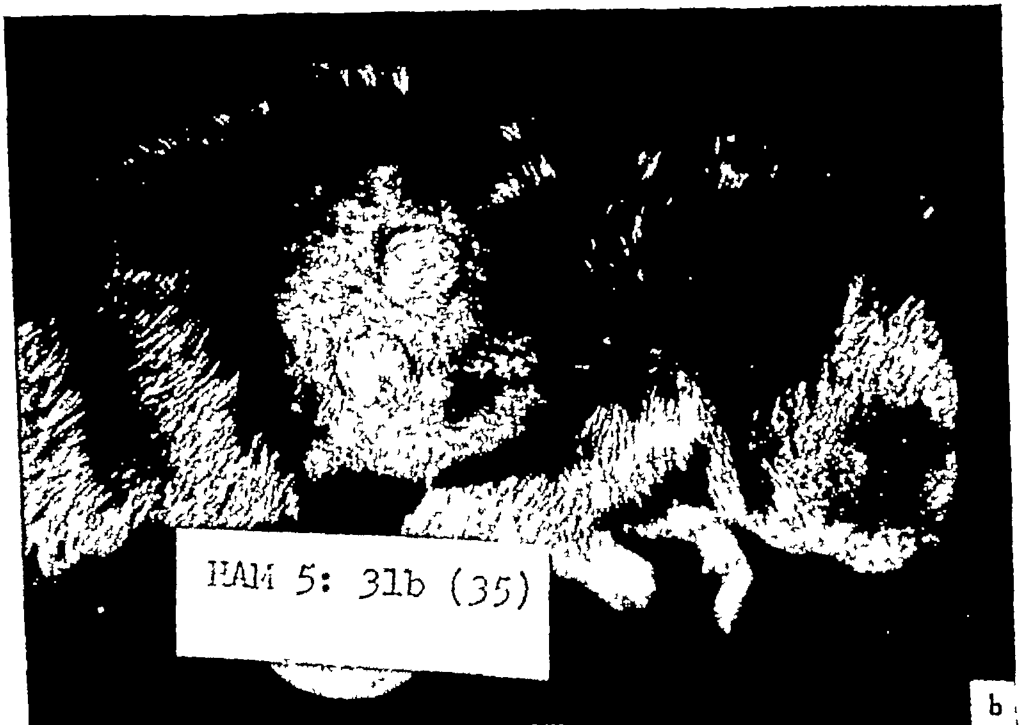
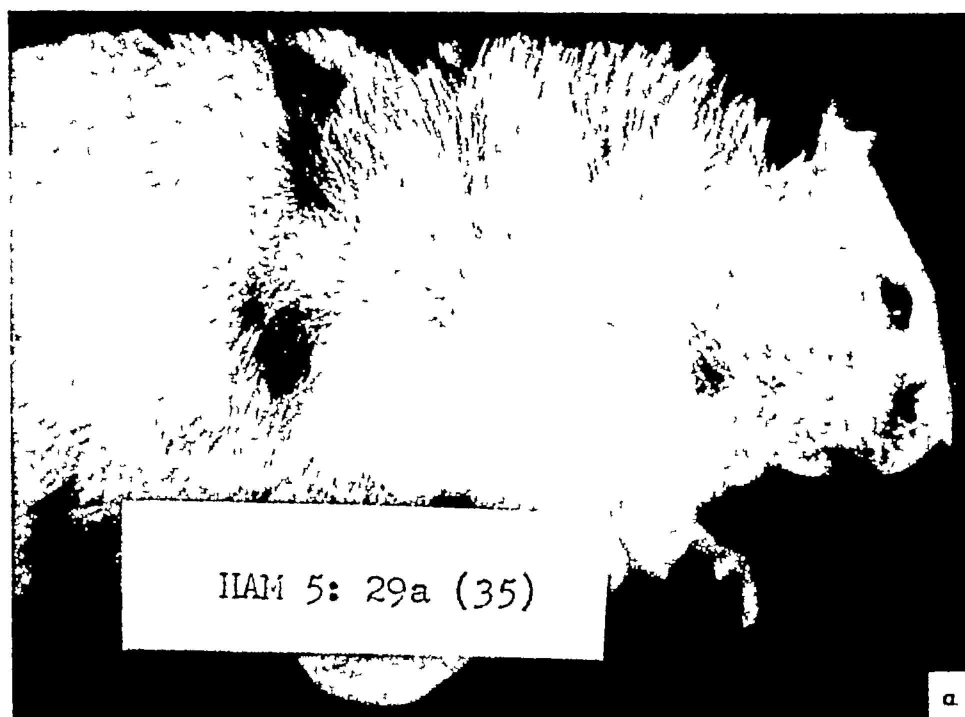




FIGURE 2 Successful intercolony homografts in normal adult Syrian hamsters, MHA → LSH (a) Albino skin grafts on agouti host 35 days after grafting Note dense crops of white hair with reversed orientation (b) The same grafts after clipping of hair



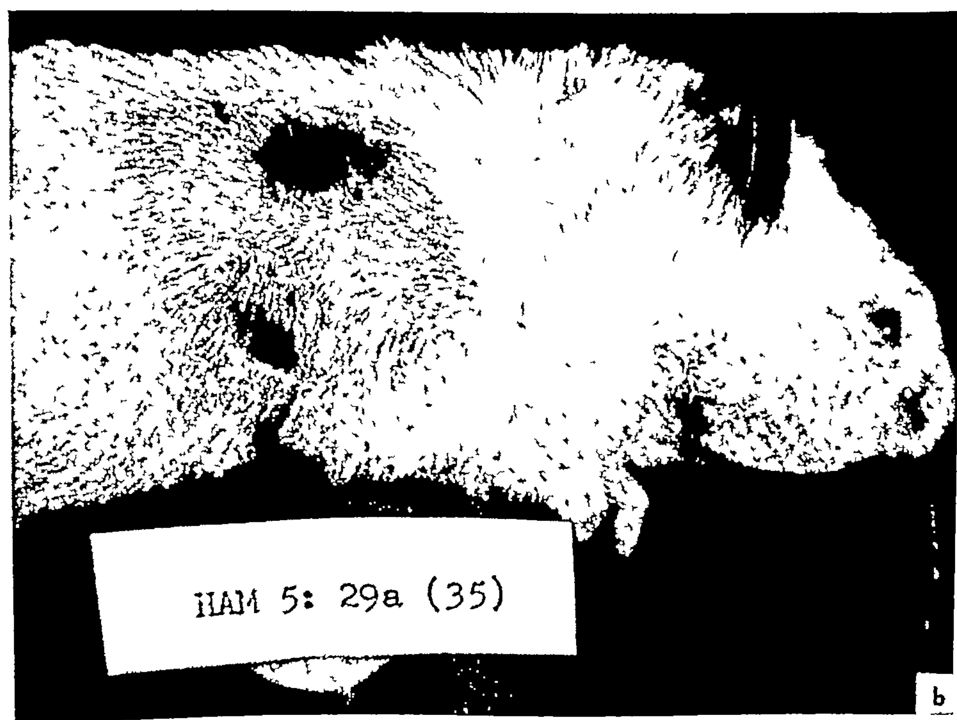




FIGURE 3 Successful intercolony homografts in normal, adult Syrian hamsters, LSH → MHA (a) Agouti skin grafts on albino host 35 days after grafting (b) The same grafts after clipping of hair

about 40 days after the initial grafting operation Although none of these animals would have been expected to reject its LSH homografts on the basis of the original intercolony tests described above, it was found that following their initial exposure to CB skin, which they destroyed, 6 of these animals did in fact eventually destroy their subsequent LSH homografts after chronic reactions From this finding it was inferred that prior grafting with CB skin must have sensitized these animals to certain weak histocompatibility antigens present in the grafts from the LSH donors

(3) Sixteen MHA hamsters that had all borne homografts from LSH donors in perfect condition for upward of 55 days were challenged with homografts from CB donors The CB grafts survived for 2 weeks or longer on 7 of the 11 MHA hosts (64 per cent) that rejected their grafts whereas, when CB homografts were transplanted to MHA hosts that had *not* previously received LSH grafts, the grafts on only one of the 14 rejectors (7 per cent) survived for longer than 12 days The median survival times of 19 3 (13 7 to 27 2) days and 10 5 (9 5 to 11 5) days, respectively, for these 2 groups of CB homografts on MHA hosts reveal that a significant prolongation of survival resulted if CB homografts were transplanted to MHA hosts already bearing LSH homografts

Although the actual proportion of MHA hosts that ultimately rejected their CB homografts was similar in both groups, none of the MHA animals reacted against its established LSH graft as a consequence of its rejection of the subsequent CB homograft This finding is rather puzzling since it has been shown, in (2), above, that the survival of LSH grafts is curtailed if they are transplanted to MHA hosts already sensitized to CB skin

*Influence of cortisone on skin graft survival* The fact that all recipients destroyed their homografts promptly in the combinations MHA → CB and CB → LSH enabled us to investigate the influence of cortisone on the survival times of skin homografts in the Syrian hamster A strong hint that this hormone would be effective came, not only from our own observation that pregnancy seemed to have resulted in prolongation of homograft survival (see the section on intercolony homografts, above), but also from numerous reports that this hormone will enable even heterografts of some neoplastic tissues to grow in this species (Toolan, 1955)

We have found with both combinations MHA → CB and CB → LSH that the subcutaneous administration of 3 mg or even 1 0 mg of cortisone acetate every third day, commencing on the day of operation, increased the survival times of the skin homografts by a factor of 2 or more in 8 of the 14 animals tested Unfortunately, this hormone had a debilitating effect on the animals Besides delaying the healing-in and vascularization of the grafts, cortisone caused a very serious loss of weight in the hosts, so that it was necessary to discontinue its administration after about the twentieth day Nevertheless, the hamsters regained their weight and normal condition very slowly, and the homografts on many of them survived for several weeks before eventually succumbing to chronic reactions Slow absorption of the deposits of cortisone under the skin, which were still visible for many days after its administration had been discontinued, may have been responsible for these long-term effects

These results show that the sensitivity of the homograft reaction to cortisone in this species is comparable to that of the rabbit.

The successful propagation of various heterologous tumors, mainly of human origin, in the cheek pouches of cortisone-treated or X-irradiated hamsters led us to determine whether cortisone would enable heterologous skin grafts from the Chinese hamster (*Cricetulus griseus*) to survive on Syrian hamsters. This particular donor species was selected since, from a purely taxonomic viewpoint, it is a much closer relative of the Syrian hamster than the donor species that have been employed in tumor heterografting experiments. In contrast with the results obtained with heterologous tumors, cortisone was completely ineffective in prolonging the survival of the skin heterografts. As with their control grafts on untreated animals, the healing-in of these grafts of Chinese hamster skin was very feeble, and the grafts remained quite indolent. None was viable beyond the sixth day.

The complete ineffectiveness of cortisone in allowing the survival of orthotopic skin heterografts, on the one hand, and its apparent effectiveness in prolonging the survival of some malignant heterografts, on the other, is rather provocative. It is possible that the cheek pouch to which most investigators have transplanted heterologous malignant tissues may be an immunologically privileged environment, where the grafts either fail to elicit a maximal response or are to some extent exempt from its consequences. Some support for this view, which is at present being investigated, may be derived from Foley and Handler's (1957) recent demonstration that cells from normal heterologous tissues will grow and proliferate for at least a short period in the cheek pouch, even without cortisone administration.

*The response of Chinese hamsters to skin homografts.* Although, apart from the Syrian hamster, many species of hamsters are known, the only species that has been domesticated and is available for study is the Chinese hamster. It is rather small and weighs about 35 gm. when full grown.

To determine whether the anomalous behavior of Syrian hamsters toward skin homografts also obtains in Chinese hamsters, we studied the fate of skin homografts in animals from a closed but noninbred colony. Of 21 animals that received skin homografts, all but 3 destroyed their grafts within 12 days. Two animals of the latter that had exchanged grafts reciprocally were peculiar in that their grafts survived for 35 and 55 days, respectively, suggesting that just by chance they were of similar antigenic constitution. The median survival time of all the homografts in this series was 8.8 (7.8 to 10.0) days. The individual survival scores and other details of various experiments are given in full elsewhere (Billingham and Hildemann, 1958).

## *Discussion*

The high degree of compatibility of skin homografts exchanged between members of the same Syrian hamster colonies is in complete accord with the findings of other workers that most spontaneous or carcinogen-induced tumors in this species survive transplantation to other adult hamsters of the same origin as the tumor donor, or even into hamsters of unrelated stocks. This

anomalous survival of both skin and tumor homografts in noninbred animals differentiates Syrian hamsters from all other mammals thus far investigated.

However, there are no grounds for postulating any deficiency in the immunological machinery of this species to account for these results. This follows from the fact that, with 4 of the 6 intercolony combinations we have tested, nearly all of the animals rejected their homografts after typical acute inflammatory reactions, and the median survival times of these grafts (that is, 8.8 to 11.2 days) fell within the range characteristic of skin homografts transplanted between individuals of distant genetic relationship in other mammalian species (Billingham *et al.*, 1954).

The recent observations of Adams *et al.* (1956) that both "intrastrain" and "interstrain" skin homografts between American-bred Syrian hamsters from commerical stocks normally survived for as long as one year are consistent with our own findings in certain colony combinations. Our present knowledge of the fate of homografts in this species suggests that in all stocks so far investigated there are only a few "strong" histocompatibility genes (antigens) segregating. Thus, the finding that all MHA hosts accepted LSH grafts, whereas the majority of LSH animals rejected MHA homografts could be explained by the segregation of one important antigen in MHA animals that was absent from LSH individuals. The situation is complicated by the fact that while all previously ungrafted MHA animals accepted LSH skin homografts, following their exposure to CB donors, no less than 60 per cent of the MHA hamsters rejected subsequent LSH grafts. This probably indicates that most CB and LSH animals share one or more "weak" histocompatibility antigens not found in MHA hamsters. This weak antigen (or antigens) was not only incapable of bringing about the rejection of LSH grafts on previously ungrafted MHA hosts, but it also failed to elicit an immune reaction against established LSH homografts when the hosts were subsequently grafted with CB skin against which they reacted vigorously. The recent work of Counce *et al.* (1956) on the role of strong and weak histocompatibility genes in the rejection of homografts in mice lends some support to this interpretation. Billingham *et al.* (1956a) have suggested that a weak level of transplantation immunity that develops slowly while a homograft is in residence may be incapable of damaging that graft, although it may effect the destruction of a subsequent homograft of the same genetic constitution during its more vulnerable healing-in phase.

Most difficult to interpret is the finding that the life expectancy of CB skin homografts may be prolonged if they are transplanted to MHA hosts bearing successful LSH homografts. Such grafts had a median survival time of 19.3 (13.7 to 27.2) days, while the control CB → MHA grafts showed a median survival time of only 10.5 (9.5 to 11.5) days. The graft survival was therefore prolonged by a factor of about 2, although 5 of 16 and 3 of 18 grafts, respectively, revealed no incompatibility even after 100 or more days. We are inclined to suspect that most of the animals in both of the donor colonies possess one or more common, weak, cellular antigens that are capable of eliciting serum antibodies. Although such antibodies appear incapable of damaging skin homografts (Billingham *et al.*, 1954, Billingham and Brent, 1956), they would

nevertheless have a specific affinity for the cells that evoked their formation In the present instance this may have resulted in an afferent or efferent interference with the immune reactions of an MHA host previously grafted with LSH skin Similar or alternative hypotheses have been suggested to account for the phenomenon of "enhancement" in mice (see Billingham *et al*, 1956b, Snell, 1956, Kaliss, 1957)

The apparent paucity of "strong" transplantation antigens in Syrian hamsters distinguishes them from all other mammals thus far studied One might well suspect that this difference is the result of an unusual stability of the histocompatibility genes Even if the individuals comprising the original family discovered in Syria in 1930 had been largely homozygous for histocompatibility genes, widespread breeding of the species during the last 20 years has provided ample opportunity for mutations to appear In contrast, minor cosmetic imperfections of immunological origin have been observed in skin homografts transplanted between sublines of highly inbred strains of mice only 8 to 13 generations apart (Billingham *et al*, 1954) It is also possible that some of the hamster homograft compatibility is a result of naturally acquired tolerance If fetal-to-fetal or maternal-to-fetal exchanges of blood cells are found to occur with any considerable frequency, one would then expect a proportion of homografts between closely related, or even unrelated, animals to be accepted for long periods or permanently There is mounting evidence that such transplacental exchanges of cells do occur in various mammals under appropriate conditions (Owen, 1956, Stormont *et al*, 1953, Levine, 1958)

To what extent homograft compatibilities in the Syrian hamster may be influenced by the interaction or penetrance of the genes responsible for isoantigens remains to be determined Indeed, the various isoantigens discovered in the present study have yet to be serologically and genetically defined Closely inbred lines would, of course, be desirable for future studies

## *References*


ADAMS, R A, D I PATT & B R LUTZ 1956 Long term persistence of skin homografts in untreated hamsters Transplantation Bull **3.** 41–42

BILLINGHAM, R E & L BRENT 1956 Further attempts to transfer transplantation immunity by means of serum Brit J Exptl Pathol **38** 566–569

BILLINGHAM, R E, L BRENT & P B MEDAWAR 1954 Quantitative studies on tissue transplantation immunity II The origin, strength and duration of actively and adoptively acquired immunity Proc Roy Soc London **B143.** 58–80

BILLINGHAM, R E, L BRENT & P B MEDAWAR 1956a Quantitative studies on tissue transplantation immunity III Actively acquired tolerance Phil Trans Roy Soc London Ser B **239** 357–414

BILLINGHAM, R E, L BRENT & P B MEDAWAR 1956b Enhancement in normal homografts with a note on its possible mechanism Transplantation Bull **3** 84–88

BILLINGHAM, R E, L BRENT, P B MEDAWAR & E M SPARROW 1954 Quantitative studies on tissue transplantation immunity I The survival times of skin homografts exchanged between members of different inbred strains of mice Proc Roy Soc London **B143** 43–58

BILLINGHAM, R E & W H HILDEMANN 1958 Studies on the immunological responses of hamsters to skin homografts Proc Roy Soc London B In press

BILLINGHAM, R E, P L KROHN & P B MEDAWAR 1951 Effect of cortisone on survival of skin homografts in rabbits Brit Med J **1.** 1157–1163

BILLINGHAM, R E & P B MEDAWAR 1951 The technique of free skin grafting in mammals J Exptl Biol **28** 385–402

COUNCE, S, P SMITH, R BARTH & G D SNELL 1956 Strong and weak histocompatibility gene differences in mice and their role in the rejection of homografts of tumors and skin Ann Surg **144** 198–204
EICHWALD, E J, C R SILMSER & N WHEELER 1957 The genetics of skin grafting Ann N Y Acad Sci **64**(5)· 737–740
FOLEY, G E & A H HANDLER 1957 Differentiation of "normal" and neoplastic cells maintained in tissue culture by implantation into normal hamsters Proc Soc Exptl Biol Med **94** 661–664
HESLOP, R W, P L KROHN & E M SPARROW 1954 The effect of pregnancy on the survival of skin homografts in rabbits J Endocrinol **10**. 325–332
KALISS, N 1957 The survival of homografts in mice pretreated with antisera to mouse tissue Ann N Y Acad Sci **64**(5). 977–990
LEVINE, P 1958 The influence of the ABO system on Rh hemolytic disease Human Biol **30** 14–28
LITCHFIELD, J T, JR 1949 A method for rapid graphic solution of time—per cent effect curves J Pharmacol Exptl Therap **97** 399–408
OWEN, R D 1956 Erythrocyte antigens and tolerance phenomena Proc Roy Soc London **B146** 8–18
PIERCE, B, E L VERNEY & F J DIXON 1957 The biology of testicular cancer I Behavior after transplantation Cancer Research **17**: 134–138
SNELL, G D 1956 The suppression of the enhancing effect in mice by the addition of donor lymph nodes to the tumor inoculum Transplantation Bull **3** 83–84
STORMONT, C, W C WEIR & L L LANE 1953 Erythrocyte mosaicism in a pair of sheep twins Science **118** 695–696
TOOLAN, H W 1955 The possible role of cortisone in overcoming resistance to the growth of human tissues in heterologous hosts Ann N Y Acad Sci **59**(3) 394–400

*Discussion of the paper*

MERRILL W CHASE (*The Rockefeller Institute for Medical Research, New York, N Y*) With reference to the very interesting work described by Billingham and Hildemann, the thought arises that the strikingly high degree of homograft tolerance among hamsters may rest on the practical absence of granulomatous responses given by this species It is known that tubercle formation and progressive disease are hardly to be encountered among hamsters when infection is undertaken with living mycobacteria Saenz[1] found that 7 hamsters of 44, treated with BCG, were capable of forming granulomas with multinucleate giant cells The observation is the more interesting when it is recalled that a granulomatous response and the production of a delayed type allergy often appear in association and that one current line of reasoning concerning homograft rejection invokes the idea of a delayed type of allergy The differences among Saenz's animals probably rest on a genetic basis

The question arises whether it would be possible to estimate, in advance of grafting, the chances for tissue incompatibility when grafts taken from hamsters of a particular colony are placed on hamsters of another closed colony Billingham's data point to the idea that genetic segregation between these "closed colonies" is a reason for the observed instances of incompatibility We deem it possible to devise some appropriate challenge that could provide a clue to the ability of hamsters to react to tissue homografts with the production of a granulomatous type of response The thought offered here is based upon the idea that hamsters may remain rather inert to the presence of tissues possessing tissue antigens differing in "individuality" unless they possess in adequate measure the capacity to respond to such antigens with a granulomatous reaction Perhaps ascertaining the histological reaction to an intracutaneous injection of paraffin oil alone, or even to paraffin oil containing a suspension of

killed mycobacteria, might reveal, between members of the hamster colony, differences of such nature that individuals most prone to form granulomas and to give other manifestations of delayed-type allergy might be ascertained be-before the time arises to use the animals either as recipients or donors of tissues

*Reference*

1 SAENZ, A 1956 Corps de Metchnikoff-Schaumann chez le hamster doré inoculé avec du BCG Ann inst Pasteur 90 751

## III. Antibodies and Antigens in Tissue Transplantation

# REACTIONS OF SKIN HOMOGRAFTS WITH SPECIFIC IMMUNE SERA

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In each of the monographs on tissue homotransplantation sponsored by this Academy[1, 2] and in various other communications on this subject, much experimental evidence has been presented indicating that the various reactions to tissue homografts have an immunological basis Some of this evidence has more specifically implicated antibodies of the conventional type in these reactions Circulating antibodies arising as a consequence of homografting have been demonstrated by Amos *et al* ,[3] Amos and Day,[4] Mitchison,[5] and others, and the biological activity of these antibodies, in certain cases at least, has been amply demonstrated The histological studies of Scothorne,[6] Darcy,[7] and others have revealed in the graft bed and regional lymph nodes the presence of an abundance of cells of the type known to be active in the production of antibody, and Darcy[8] has shown a nice correlation between the number and time of appearance of these cells and the rejection of first-set and second-set homografts The prolonged survival of homografts in animals treated by means known to suppress antibody formation and the long survival of homografts in patients with agammaglobulinemia both suggest that conventional antibody is necessary if not sufficient for homograft rejection

If these considerations were reinforced by the ability of immune sera passively to transfer specific homograft immunity, there would be little difficulty in bringing the whole body of transplantation phenomena into the realm of classic immunology However, while such transfer has, in fact, been abundantly demonstrated in the case of certain tumor grafts,[4] the results of similar experiments with skin-graft immunity have usually been negative While Voisin and Maurer[9] obtained suggestive results in some guinea pigs, the general experience of Billingham *et al*[10] and others has been that serum from skin-graft-immune animals does not passively transfer the state of immunity

Our working hypothesis, based chiefly on histological considerations, is outlined in FIGURE 1 As the time of homograft rejection approaches, large numbers of plasma cells can be distinguished in the graft bed[8] and in the regional lymph nodes[6] If one assumes that these cells are engaged in producing antibodies to graft antigens, then it follows that the concentration of these antibodies would be higher at the site of their production than elsewhere in the host (in the blood stream, for example) Much of the antibody that reaches the graft and is there bound to graft antigens might arrive by diffusion through tissue spaces in the graft bed rather than by the returning circulation

Attempts at reproduction of this situation by passive transfer of serum might then fail for simply quantitative reasons, whereas the local injection of a high-titered serum around a graft might more closely simulate the actual state of affairs

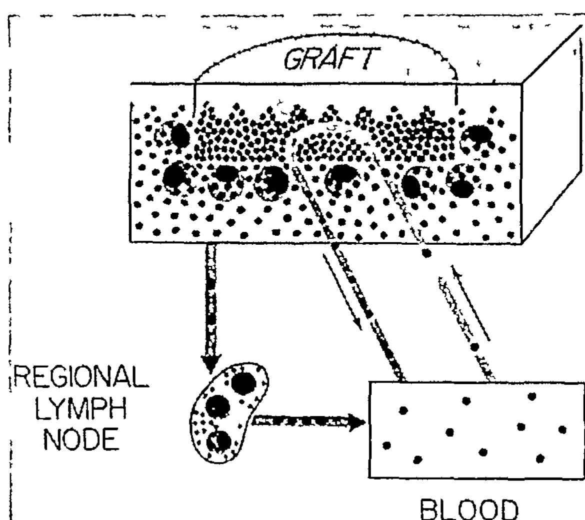



FIGURE 1 Schematic representation of local antigraft antibody formation by plasma cells in the graft bed and regional lymph nodes The black dots represent hypothetical relative antibody concentrations and indicate that the amount of antibody fixed to graft antigens may be large compared to the amount in serum

Most passive-transfer experiments in the past have been done with animals made putatively immune by the application of skin homografts, a procedure that would not necessarily be expected to be the most effective means of immunization Medawar[11] has shown that transplantation immunity can be induced effectively by the injection of suspensions of leukocytes or spleen cells, and this finding has suggested the possibility that more potent antisera might be obtained by incorporating such cell suspensions in the well-known Freund's adjuvant mixture

Hybrid rabbits, immunized against normal rabbit spleen with adjuvants, have been found uniformly to develop individual-specific "white-graft" immunity within two weeks, that is, homografts from the donor of the spleen cells fail to be vascularized and eventually dry up, while control homografts from other rabbits are vascularized and rejected in the normal fashion Similar results were obtained in A/Jax mice immunized against BALB/c spleens, in this case the white-graft immunity being strain specific rather than individual specific In both rabbits and mice this white-graft immunity has been readily transferred with serum, that is, when serum from an immunized animal bearing a white graft is injected into a normal animal, the subsequent application of a skin homograft from the spleen-cell donor is followed by a white-graft reaction An illustration of such a passive white-graft reaction is given in FIGURE 2 Both mice had received homografts 8 days previously, and the mouse at the bottom had simultaneously received intravenously 0 4 ml pooled serum from specifically immunized mice The homograft in the control mouse has vascularized and is now exhibiting the characteristic hemorrhagic rejection reaction, while the passively immunized mouse shows a typical white graft

Such grafts are nonvascularized, as judged by various criteria, including failure to bleed when pricked or sliced They may persist as soft intact grafts



FIGURE 2 Passive transfer of "white-graft" immunity The grafts were applied 8 days previously, and the top (control) homograft shows beginning hemorrhagic necrosis The mouse below had received 0 4 ml pooled immune serum at the time of grafting, the graft is not healed and is nonvascularized

for several days beyond the expected rejection date before finally drying up and sloughing

It might be well at this point to suggest that the white graft has perhaps been underemphasized in transplantation literature There seem to be good reasons for regarding it as the expression of a higher degree of transplantation immunity than the usual second-set response For instance, in the latter response the accelerated rejection is in fact preceded by an initial period of acceptance and vascularization, while the white graft is not tolerated to this extent [10] Heterografts of guinea pig or mouse skin to rabbits typically become white grafts, and this reaction is commonly regarded as intolerance of some higher order than that shown to homografts Second-set homografts applied at the height of the reaction to first-set grafts are also likely to become white grafts, but if one waits for 2 weeks or more, until the reaction to the first-set graft has sub-

sided, the second-set graft usually will be accepted temporarily and then rejected in an accelerated fashion [12]

From what is known of the antibody response to homografts, and from the results of the experiments just reported, it would appear that the white-graft type of reaction results from the application of a homograft at a time when the serum contains a sufficiently high titer of specific antigraft antibodies It may be that under these circumstances the failure of the graft to become vascularized is the result of the destruction of ingrowing capillary sprouts by Arthuslike reactions as they carry circulating antibody into intimate contact with the graft Another possibility, of course, is that the graft may be quickly and directly damaged by cytotoxic antibody, as in the experiments of Billingham[13] with epidermal cells and of Mitchison and Dube[14] with homologous tumor cells

If the white-graft reaction is an expression of the immune state itself, then the accelerated or second-set reaction may be likened to the "secondary re-



FIGURE 3 Specific homograft rejection produced by local injection of immune serum Photograph of homografts from unrelated rabbits on the sixth day after grafting and 48 hours after the local intradermal injection of pooled immune serum produced against spleen cells of the donor of the homograft on the right The control graft is in good condition, while the test graft has been rejected

sponse" of classic immunology The initial acceptance and vascularization of the second-set graft may well be occurring in the virtual absence of circulating antibody, with the subsequent rejection resulting from rapid and copious local formation of antibody If this interpretation is correct, then attempts passively to transfer this kind of reactivity with serum would be expected to be negative, as the capacity of an animal to exhibit a secondary response cannot, of course, be transferred in this manner However, one might expect to be able to produce accelerated rejection in a vascularized homograft by supplying antibody in sufficient amount We have attempted this, using individual-specific sera in rabbits and strain-specific sera in mice, as described earlier A typical experiment consists of the application of a test and a control homograft to the ears of a normal rabbit After 2 to 4 days have elapsed to permit vascularization, immune serum is injected in small quantities in multiple sites around each graft On occasion, typical hemorrhagic necrotic rejection reactions have occurred in the test homografts (FIGURE 3) In spite of a considerable amount of work, it has not been possible thus far to develop a method for reproducibly eliciting this phenomenon, and only 10 serum pools of approximately 150 have been active However, the reaction appears usually within 24 hours after the serum injection, and sometimes within 6 hours, and the reactions have always been specific, that is, the control homografts have never been demonstrably affected

Our conclusions from these experiments are that vigorous immunization of rabbits or mice against homologous spleen cells results in the development of specific transplantation immunity of the white-graft type, that this immunity can be transferred passively with serum from these immunized animals, and that such sera, upon local injection, can also cause accelerated rejection of specific homografts

The significance of these observations with respect to the classic homograft reaction must remain in doubt, since demonstations that antisera can cause homograft rejection are by no means proof that this is the mechanism actually in operation Final clarification of this point may well await a better understanding of the nature of the antigens and antibodies involved in these phenomena

*References*


1 ANNALS OF THE NEW YORK ACADEMY OF SCIENCES 1955 The Relation of Immunology to Tissue Homotransplantation 59(3) 277–466

2 ANNALS OF THE NEW YORK ACADEMY OF SCIENCES 1957 Second Tissue Homotransplantation Conference 64(5). 735–1073

3 AMOS, D B, P A GORER, Z B MIKULSKA, R E BILLINGHAM & E M SPARROW 1954 An antibody response to skin homografts in mice Brit J Exptl Pathol **25**· 203

4 AMOS, D B & E D DAY 1957 Passive immunity against four mouse leukoses by means of isoimmune sera Ann N Y Acad Sci **64**(5) 851–858

5 MITCHISON, N A 1955 Isoantibody against a tumor Transplantation Bull **2**. 93

6 SCOTHORNE, R J 1957 Studies on the response of the regional lymph node to skin homografts Ann N Y Acad Sci **64**(5)· 1028–1039

7 DARCY, D A 1949 Plasma cells in reactions against rabbit tissue homografts Nature **163** 98

8 DARCY, D A 1952 Phil Trans Roy Soc London **B236** 463

9 VOISIN, G A & P MAURER 1957 Studies on the role of antibodies in the failure of homografts Ann N Y Acad Sci 64(5). 1053–1073
10 BILLINGHAM, R E, L BRENT & P B MEDAWAR 1954 Quantitative studies on tissue transplantation immunity II The origin, strength and duration of actively and adaptively acquired immunity Proc Roy Soc London **B143** 58
11 MEDAWAR, P B 1946 Immunity to homologous grafted skin II The relationship between the antigens of blood and skin Brit J Exptl Pathol **27.** 15
12 RAPAPORT, F T & J M CONVERSE 1958 The immune response to multiple set skin homografts an experimental study in man Ann Surg **147** 273–280
13 BILLINGHAM, R E 1957 Studies on epidermal cell suspensions, with particular reference to problems of transplantation immunity Ann N Y Acad Sci **64**(5) 799–810
14 MITCHISON, N A & O L DUBE 1955 Studies on the immunological response to foreign tumor transplants in the mouse J Exptl Med **102** 179

## *Discussion of the Paper*

MERRILL W CHASE (*The Rockefeller Institute for Medical Research, New York, N Y*) I should like to ask Stetson, in connection with his very interesting work, first whether he has any particular knowledge concerning the 10 active serum pools of the total of 150 pools that he mentioned? Second, are these special pools consistently active? Finally, has he gained further knowledge of, or has he the expectation of investigating, the antibody content within the circulation of animals that have rejected homografts? If his theory of a particularly high local production of antibody in the immediate vicinity of migrating white cells at the base of the graft is correct as the chief cause for homograft rejection, then spillover of this antibody or antibodies into the general circulation should result in a detectable concentration in the serum following normal homograft rejections

CHANDLER A STETSON, JR We know very little about the antibody content of our active sera This, of course, is due to the fact that the nature of the tissue antigens involved in homograft immunity is not clear at present Perhaps the hemagglutinins described by Gorer[1] and others in the sera of grafted animals are responsible for the antigraft reactions that we have been studying We certainly intend to pursue this aspect of the problem further

## *Reference*

1 GORER, P A. 1956 Some recent work on tumor immunity Advances in Cancer Research 4· 149–186

# THE VASCULARIZATION OF SKIN HOMOGRAFTS AND TRANSPLANTATION IMMUNITY*

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The properties of the homograft-rejection reaction show close similarities to the events observed in the secondary responses of orthodox immunological systems After rejection of a homograft from one donor the host has acquired a sensitivity directed specifically against further applications of tissues from that donor If a second homograft from this same donor is now applied, it is destroyed in an accelerated fashion (the second-set phenomenon) [1, 2] Whereas the first-set homograft is rejected within 7 to 10 days after application, second-set grafts seldom survive beyond the fourth or fifth postoperative day Additional repeat-set homografts show no further shortening of the recipient's rejection time, but are destroyed at the same rate as the second-set grafts [3, 4]

A peculiar type of secondary response occurs when a second-set homograft is transplanted within seven days after the rejection of a first-set homograft The vascularization of the graft is defective, and the transplant retains a white color This "white-graft" reaction, observed in man[4] and confirmed in the rabbit by Stetson and Demopoulos elsewhere in this monograph, is attributed to an inability of the host vessels to connect with the graft vessels and to penetrate the graft during the period of the maximum response of the host to the first-set homograft

This report deals with another aspect of the homograft reaction, namely, how soon after transplantation of a skin homograft does transplantation immunity develop? How advanced must the graft-host connection be before the antigenic stimulus reaches the host?

In the early stages after transplantation during the so-called period of "plasmatic circulation" there are indications of uptake by the graft of fluid and cells, as suggested by early color changes and increase in weight of mammalian skin grafts placed on the chorioallantois of the chick embryo [5] It is generally assumed that exchanges between the graft and host do not occur until a period of approximately forty-eight hours has elapsed, that is, after the establishment of the first connections between blood and lymphatic vessels of the graft with those of the host The development of systemic specific sensitivity to a skin homograft, therefore, might be expected to occur only after this time interval of approximately forty-eight hours The experiments described in this report were designed to confirm this assumption

The second-set phenomenon, the accelerated rejection of a second homograft from the same donor, is at present the only indicator of the development of transplantation immunity

The experimental protocol employed first- and second-set homografts placed

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on the auricle of the rabbit After varying time intervals of from two to nine days, the first-set homograft was excised from the host by amputation of the recipient auricle A similar principle was applied by Freund and Lipton,[6] who excised the site of an intracutaneous injection of spinal cord suspension in water-in-oil emulsion containing killed tubercle bacilli one hour after the injection They noted that the excision did not prevent either the development of allergic encephalomyelitis or sensitization to tuberculin, as a portion of the injected material reached the regional lymph nodes within one hour

### *Methods and Techniques*

All animals used were female rabbits weighing from 2 5 to 3 0 kg They were grouped into 5 consecutive series of 10 to 12 rabbits per donor Each donor was obtained from a different source in order to avoid the possibility of genetic similarity to the recipients One half of each group of recipients was sensitized by a first-set skin homograft placed on the right auricle, the other half of each group received the first-set homograft on the left auricle (FIGURE 1) At varying time intervals after transplantation of the first-set homografts the auricle containing the homograft was amputated A second-set homograft from the same donor was then placed on the homolateral auricle (FIGURE 1) The criterion of the development of transplantation immunity in the host was the occurrence of a second-set phenomenon in the second-set homograft In order to avoid a white-graft reaction, a time interval of 17 days was allowed between the first- and second-set grafts

After time intervals of 2, 3, 5, 7, and 9 days for each series, the auricle containing the first-set graft was amputated Seventeen days after transplantation of the first-set homograft, the recipients received their second-set homograft on the homolateral auricle In all cases the same donor supplied the first- and second-set skin grafts

The auricles of both recipient and donor were clipped (not shaved) and

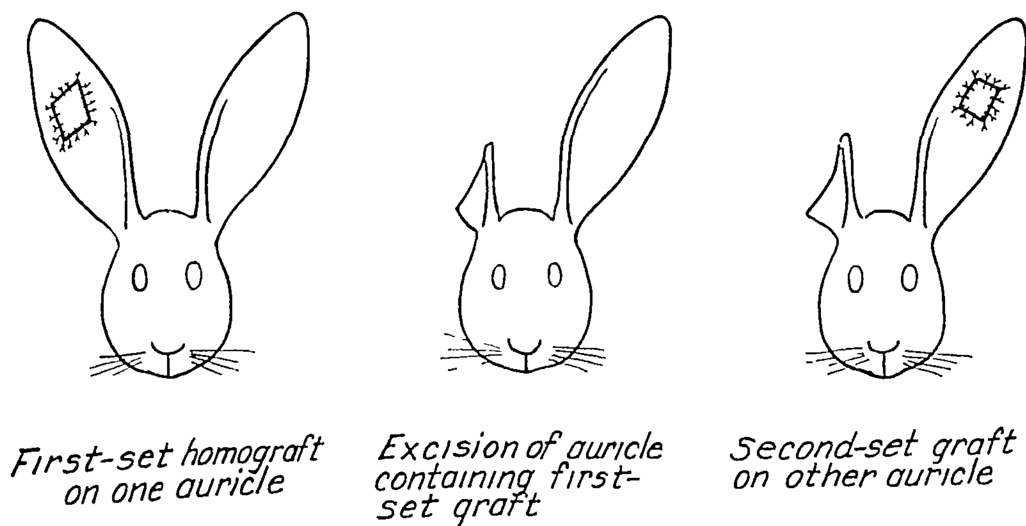



FIGURE 1

TABLE 1

| Group number | Number of rabbits per group | Timeline (first-set homografts on day 0; excision of first-set homografts; second-set homograft on day 17) | Mean survival time in days of second-set grafts |
|---|---|---|---|
| I | 16 controls | 0 ---- No excision ---- No second-set ----→ • (day 17) | 7 9 |
| II | 19 | 0 … • Second day postoperative ←—— 15 days ——→ • | 7 6 |
| III | 18 | 0 … • Third day postoperative ←—— 14 days ——→ • | 5 9 |
| IV | 10 | 0 … • Fifth day postoperative —— 12 days ——→ • | 5 5 |
| V | 12 | 0 … • Seventh day postoperative ←—— 10 days ——→ • | 5 2 |
| VI | 19<br>10 in winter<br>9 in spring | 0 … • Ninth day postoperative ←—— 8 days ——→ • | 4 4<br>4 7 in winter<br>4 1 in spring |

Day 0 1 2 3 4 5 6 7 8 9 17

First-set homografts (↑ day 0)

Excision of first-set homografts (↑ days 2, 3, 5, 7, 9)

Second-set homograft on seventeenth day after first-set (↑ day 17)

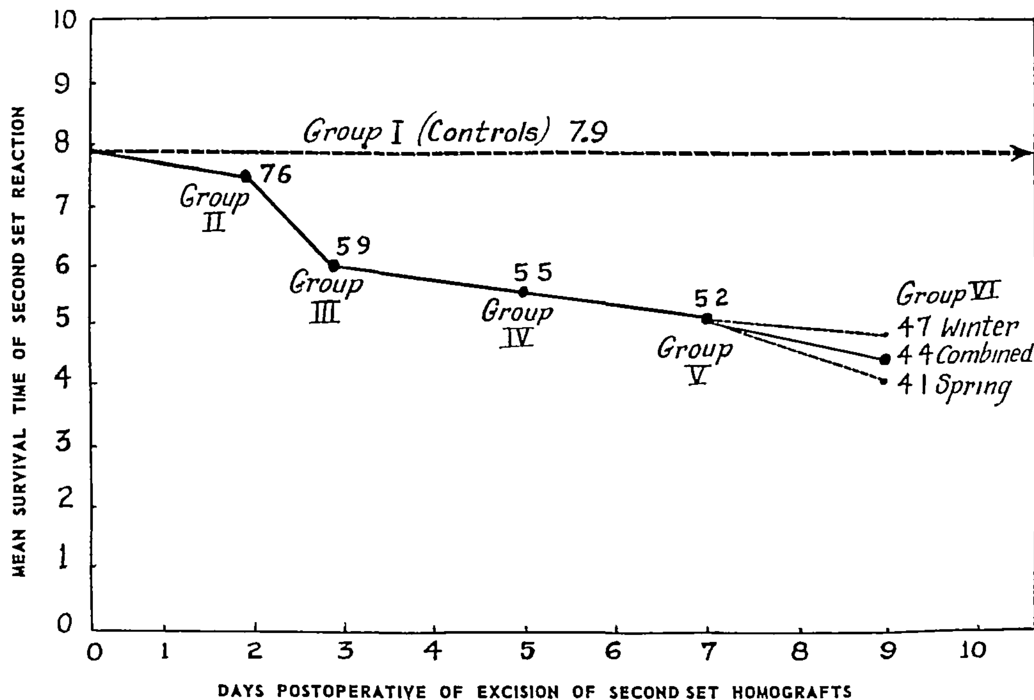



FIGURE 2

washed with 70 per cent alcohol immediately before surgery The auricle of the donor rabbit was amputated at the junction of the medial and the distal thirds, the resected auricular segment served to supply 12 skin grafts After trimming the margins of the resected segment of auricle the skin on the ventral aspect was carefully removed from the perichondrium to provide the skin grafts The dermal surface of the skin was then spread over a moistened sterile paper towel With the paper towel as a backing, the skin was cut into 12 pieces of 15 × 10 mm in dimensions A rectangular area 10 × 15 mm was outlined and incised in the ventral surface of the recipient auricle The skin was removed from the perichondrial bed with little attendant bleeding If bleeding persisted, it was controlled by light compression with moistened gauze

All controls received only one set of skin homografts In a number of series of grafts the experiment was repeated with additional 10 to 12 recipients and one donor to verify the original findings

Daily observations of the second-set grafts were made through a film of mineral oil under a stereo-microscope,* following the technique of Taylor and Lehrfeld [7] The time of skin rejection was determined when complete vascular disruption was observed in the homografts

## *Results*

As illustrated in TABLE 1, the controls and the groups of 5 consecutive series of rabbits were assigned Roman numerals as following Group I (controls), Group II (2-day interval), Group III (3-day interval), Group IV (5-day interval), Group V (7-day interval), and Group VI (9-day interval)

* Product of Bausch & Lomb Optical Co , New York, N Y

It is of interest to note that the time of onset of the homograft rejection period in the rabbits of Group II (2-day interval) coincides closely with that of Group I (controls) The data in TABLE 1 show that the mean survival times of the homografts in the Group I (controls) and Group II (2-day interval) were 7 9 and 7 6 days, respectively There was a considerable decrease in the homograft rejection period, with a mean survival time of 5 9 days in the Group III (3-day interval) This indicates that the earliest development of transplantation immunity as indicated by the second-set phenomenon occurs after the time interval of 48 hours

TABLE 1 and FIGURE 2 show that there is a progressive decrease in the time of onset of the second-set homograft rejection period with the increasing time intervals Data illustrated in TABLE 1 show that the mean survival times of the second-set homografts in the following series are 5 5, 5 2, and 4 4 days in Group IV (5-day interval), Group V (7-day interval), and Group VI (9-day interval), respectively

The second-set homografts of Group VI (TABLE 1) seem to survive somewhat longer during the winter than during the summer

Postoperative observations showed that a few of the second-set homografts in all series had active hemal flow in the blood vessels by the second day This was not observed in the controls receiving only one homograft (Group I)

## *Conclusions*

The establishment of vascularization of a skin homograft is required for the development of transplantation immunity and the hypersensitive state The presence of the skin homograft in the host bed for a period of at least forty-eight hours is required The degree of hypersensitivity developed by the host is progressive, increasing as the vascular connections become better established

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## *References*

1 MEDAWAR, P B 1945 A second study of the behaviour and fate of skin homografts in rabbits J Anat **79**· 157
2 ROGERS, B O 1957 The genetics of skin homotransplantation in the human Ann N Y Acad Sci **64**(5). 741
3 LEHRFELD, J W, A C TAYLOR & J M CONVERSE 1955 Observations on second and third set skin homografts in the rat Plastic and Reconstructive Surg **15.** 74
4 RAPAPORT, F T & J M CONVERSE 1958 The immune response to multiple-set skin homografts An experimental study in man Ann Surg **147** 273
5 CONVERSE, J M, D L BALLANTYNE, B O ROGERS & A P RAISBECK 1957 Plasmatic circulation of skin grafts Transplantation Bull **4:** 154
6 FREUND, J & M M LIPTON 1955 Encephalomyelitis after excision of injection site J Immunol **75** 454
7 TAYLOR, A C & J W LEHRFELD 1953 Determination of survival time of skin homografts in rat by observations of vascular changes in graft Plastic and Reconstructive Surg **12**· 6

# HOST-GRAFT INTERRELATIONSHIP AND THE EFFECTS OF INJECTIONS OF ORGAN HOMOGENATES AND OF CELLS UPON THE TESTES OF EXPERIMENTAL ANIMALS*

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## Introduction

Since 1945, problems concerned with the host-graft interrelationship have occupied my attention and, in a series of publications,[1-5] I have described experiments in which a variety of transplants of organs were made to the seminal vesicle in rats This work demonstrated that autografts of adrenal cortex and homografts of ovary and corpus luteum were maintained sufficiently well in turn to stimulate the secretory epithelium of the seminal vesicle of the castrated host, indicating that andromimetic substances were secreted by the grafts Testicular autografts and homografts also were capable of sustaining the epithelium of the seminal vesicle, but the germinal portions of these grafts gradually degenerated, presumably because of the intra-abdominal hyperthermic environment Thyroid autografts in the seminal vesicle continued to secrete hormone, judging by the cytology of the follicular epithelium and the presence of colloid, but these grafts did not stimulate the vesicular epithelium Other organs (for example, the pancreas and the salivary glands) became atrophic

It was noted also that successful grafts of tissues, such as the adrenal, ovary, and testis, were essentially free of lymphocytes, macrophages, or other cells known to be associated with antibody formation or transport or with antigen sequestration Similarly, the seminal vesicles were also devoid of these cells, which hereafter will be referred to as the "cells of the antibody series" Since the seminal vesicles normally are free of cells of the antibody series, it seemed that this organ should receive attention as a preferred site for transplantation

As an outgrowth of these experiments it was of interest to determine whether grafts could induce organ-specific damage in hosts in which the target organ was not removed In the background was the thought that each organ, during differentiation and maturation, acquired unique molecular properties that normally conditioned its own size and function and that, as a result of damage such as injury or infection, caused self-destruction or overgrowth Would it be possible, then, to induce overgrowth or destruction of a specific organ by presenting the host with the appropriate substrate in the form of a transplant? To examine this possibility, it was decided to devote attention to one organ, the testis, because antigens to it had been known for some time (see Smith[6] and Katsh[7] for partial reviews) to be present in testis or sperm A series of experiments was undertaken during the next several years to examine the possibility of affecting selectively the testes of rats by autologous and homologous grafts of testes Since problems associated with transplants of endocrine tis-

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sues were recognized, a variety of experimental conditions, such as X irradiation, vitamin imbalance, and hypothermia, was employed The experiments were inconclusive, and the intended goal was not attained, but other data[8–12] obtained during these studies are of continuing interest and may be related to the problem at hand in future studies

Although these efforts failed to test the possibility of inducing organ-specific effects in hosts by means of homologous organ transplants, enthusiasm for the line of investigation did not wane With specific reference to the testis, there was evidence in the literature[13, 14] indicating that injections of sperm or testis could induce spermatogenic degeneration It seemed worthwhile, therefore, to gain an appreciation of the reasons why injections of material were effective and transplants were not, and it was decided to examine the problem from the former aspect in order to gain information pertinent to transplantation studies The remainder of this presentation is concerned with the experiments using injections of testicular materials

One set of data is concerned with the effects of homologous testicular and brain and heterologous testicular homogenates in adjuvant in the guinea pig[15] It was observed that homologous testicular material was more efficient than homologous brain or heterologous testis in inducing aspermatogenesis The order of efficiency is presumably related to the degree of cross-reactivity of the injected material with the testicles of the host and, therefore, similarity or dissimilarity of the injected antigen to the native antigen Another important part of the experiments permitted the conclusion that the native antigen was localized solely in the spermatogenic tissue and was not present in the nongametogenic portions of the testes Other experiments employing a variety of tissues as antigens and experimental animals as hosts[16] indicated that the rat and rabbit were not as sensitive as the guinea pig in responsiveness to the organ or cell injections It was decided, therefore, to concentrate on the guinea pig as the test animal and to examine as many as possible of the reactions of the animal to injections of sperm and testis

The reactions of the female guinea pig to injections of homologous testis or sperm with and without adjuvant were examined The female, rather than the male, was used in order to remove the possiblity that endogenous antigen would lead to equivocal findings In this set of experiments[7, 17] the injections, whether in adjuvant or in saline, induced a hypersensitive state of the anaphylactoid type as measured by the responses of isolated uteri and ilea to homologous and heterologous sperm *in vitro* The term anaphylactoid rather than anaphylactic is used for reasons stated elsewhere[17] and because several injections of sperm or testis in saline[7, 13, 15, 17] did not cause anaphylaxis, whereas even a single injection of egg albumin into the immunized guinea pig results in true anaphylaxis[18] Freund *et al*[19] demonstrated that by chemical extraction of testicular tissue an antigen was obtained which, upon injection into the guinea pig, induced anaphylactic systemic and skin reactions as well as true anaphylaxis The same workers reported, however, that systemic anaphylaxis or skin reaction was not demonstrable by the use of sperm or testicular suspensions

As a result of the demonstration of the anaphylactogenicity of sperm, it was necessary to examine the possibility that aspermatogenesis might be induced by an anaphylactoid response of the vas deferens A closure of the lumen of the vas deferens, preventing the progress of spermatozoa, might result in destruction of the spermatogenic tissue If this was the mechanism of the induction of aspermatogenesis, the concept of organ damage by homologous-organ antigen would be rudely jolted This possibility was examined in the next series of experiments [20] The studies *in vitro* performed on the organs of guinea pigs injected with homologous testis, sperm, and ovalbumin demonstrated that, whereas the isolated ileum and uterus always responded to specific antigen *in vitro,* the vas deferens and seminal vesicle did not The reason for the insensitivity of the vas deferens and seminal vesicle is related to the absence in these organs of cells of the antibody series Because the vas deferens failed to show anaphylactoid response, the possibility that the mechanism of aspermatogenesis could be attributed to this phenomenon seemed remote The situation *in vivo,* however, may be quite different from that *in vitro* because, in the former case, cells of the antibody series probably course through the organ continuously in the blood vessels Nevertheless, the literature pertaining to the effects of ligation of the vas deferens indicates that spermatogenic tissue is affected only slightly, if at all This, together with the evidence presented above and elsewhere,[15] permits the deduction that the damage observed in the testes of the guinea pigs following injections of sperm or testicular cells is related to an immune phenomenon, leaving the concept unviolated

The evidence that the seminal vesicles are devoid of cells of the antibody series supports the suggestion that the seminal vesicle (like the anterior chamber of the eye and the brain[21]) may be a preferred site for transplantation In contrast, the ileum, uterus, and skin, in which lymphoid elements and macrophages are found normally in great numbers, are prepared to reject grafts and to show organ anaphylaxis promptly This will be considered later in the presentation of a unified hypothesis that integrates information gained in experiments on transplantation, anaphylaxis, and such isoallergic disease states as uveitis,[22] neuritis,[23] and encephalomyelitis,[24] as well as aspermatogenesis

Now a series of experiments relating to the isoallergic diseases must be discussed

The experiments concerned with the contribution of the bacterial component of adjuvant that conditions the sensitization of the animal and its organs will be mentioned only briefly here because of their extensive nature The full report will appear subsequently

It will be recalled that many injections of sperm or testis suspensions in saline are inefficient[13, 15] by comparison with a single injection of such materials in complete adjuvant (paraffin oil, Arlacel A, and *Mycobacterium butyricum*) in the induction of aspermatogenesis [14, 15] Similarly, one or more injections of sperm or testis in incomplete adjuvant (paraffin oil and Arlacel A, but no bacteria) have no pronounced effect upon the testes of guinea pigs [14, 15] Apparently, then, the bacteria in adjuvant are important in the mediation of the syndrome Moreover, because it appeared that only acid-fast bacteria had been employed successfully in adjuvants, it seemed that acid-fastness of the

bacteria must receive attention Therefore, adjuvants prepared with acid-fast and with nonacid-fast bacteria were emulsified with homologous testis and injected into male guinea pigs The testes were examined histologically, and the organs were tested *in vitro* as before The testes of animals injected with the acid-fast bacterial preparation were aspermatogenic, and these animals also had ilea that responded strongly to antigen *in vitro* In animals injected with the nonacid-fast bacterial mixtures, only mild, if any, testicular damage was observed, and the isolated ilea reacted weakly (in *duration* of contraction, not as regards *height* of contraction) Another experiment related acid-fastnesss and ability to induce aspermatogenesis when, used in the above manner, *M tuberculosis* from which acid-fast stainability was removed was completely ineffective in inducing aspermatogenesis and ileal sensitivity A further series of experiments disclosed that lipopolysaccharides extracted from virulent strains of *M tuberculosis* were extremely effective in inducing aspermatogenesis and ileal responsivity when incorporated into adjuvant and injected in the manner described above Lipopolysaccharides from nonacid-fast bacteria were considerably less potent in these respects

The working hypothesis can now be presented in outline form and a detailed elaboration supported by the appropriate evidence attained to date will be presented in future publications

## Host, Tissue, and Organ Responses in Anaphylaxis, Transplantation, and Other Induced or Acquired Hypersensitive States in Which Organ-Specific or Cell-Specific Damage is Produced

### *Anaphylaxis*

*Portals of entry of antigen and host response to the sensitizing dose of antigen* When the sensitizing exposure to antigen is intracutaneous (by way of injection, cutaneous damage, or infection), macrophages, lymphocytes, and other white blood cells accumulate at the site The macrophages transport antigen to other foci of lymphoid tissue, synthesis of antibody is promoted thus, not only in the skin, but also elsewhere in the body

A similar response occurs if other portals of entry that contain macrophages and lymphocytes, such as the respiratory passages and alimentary tract, are originally involved The net result is that the tissue itself, as well as other organs containing lymphoid tissue, may become sensitized

An interval of time subsequent to the exposure to the sensitizing dose is required for antigen transport to the individual organs that contain foci of lymphoid tissue and antibody synthesis in them After this time (up to 3 weeks), little or no detectable antibody may be present in the circulating blood because the major concentration of antibody-containing cells remains in the individual organs Consequently, the blood levels of antibody reflect only the amount of circulating lymphocytes as they are released from the lymphoid centers At this time and later, however, a provoking dose of antigen will elicit the following responses, depending upon the organ, the route of administration, the amount of antigen, and other factors, such as the rapidity of antigen administration and the vehicle used

*The skin* A challenging dose of antigen in the skin elicits a cutaneous re-

sponse by virtue of the interaction of the antigen with the lymphocytes and plasma cells (cells of the antibody series) During this interaction there is liberated a factor that is responsible for the local wheal and flare reaction

If the amount of antigen or the rapidity of its administration during the challenging phase is such that all the units of antigen can be "neutralized" (that is, reacted with) by the lymphocytes in the skin, free antigen will not be available to react elsewhere Conversely, if the amount or rapidity of antigen administered exceeds the available number of cells of the antibody series at the site, excess antigen will pass to other organs of the body

Similar reactions occur if the portals of entry of the challenging antigen are the respiratory passages or gut

*Organs containing smooth muscle and lymphoid tissue, such as the ileum, the uterus, and the respiratory passages* As a result of a sufficient sensitizing exposure, antigen is carried to other organs of the body There are discrete lymphoid follicles in the ileum and respiratory passages, and the uterine mucosa is infiltrated with lymphocytes Antigen brought to these organs stimulates the production of antibodies in the lymphocytes therein The administration of a challenging dose of antigen to these organs results in antigen interaction with the lymphocytic cells This interaction is followed by the release of a factor that stimulates the contraction of the smooth musculature of these organs The contraction of the smooth musculature may be brief or prolonged, depending upon the number of lymphocyte-antigen interactions If the number of antigen units is large and all available antibody-containing cells have an opportunity to react, the amount of factor released is large and the contracture is of long duration After this, the organ is desensitized and will not respond to further antigen If the amount of antigen is small, but the number of antibody-containing cells is greater, the organ will not be desensitized, and further exposures to antigen will induce contractions until all antibody-containing cells are exhausted

It can be seen, therefore, that a challenging dose of antigen that must escape neutralization at the portal of entry may have delayed effects on other organs elsewhere Indeed, the amount of sensitizing antigen that escapes to the various organs dictates the degree of sensitization of that organ, and the amount of challenging antigen that escapes to the organ dictates the degree of response By intravenous injection of an adequate amount of the challenging antigen, the antigen is presented to all sensitized organs rapidly, so that death may ensue

*Organs containing smooth muscle, but no lymphoid tissue the vas deferens and the seminal vesicle* In organs such as the vas deferens and seminal vesicle that contain smooth muscle, but no accumulation of cells of the antibody series, the sensitizing antigen has no effect in the absence of lymphocytes Therefore, exposure of these organs to the challenging antigen does not provoke contracture unless lymphocytes happen to be coursing through the vessels in the organ In the latter instance, the response of the musculature is related to the number of antibody cells present at a given time that react with the antigen In actual experimentation, three instances (out of more than one hundred trials) of minute

responses to antigen *in vitro* were observed. The three instances of response can be attributed to lymphocytes present in the vessels of the segment *in vitro*. Mast cells were not involved in the responses of the seminal vesicles or vas deferens. It is suggested, therefore, that the factor liberated during antigen-antibody cell interaction is not histamine, even though release of histamine may be a concomitant feature of such reactions.

*Organs containing neither smooth muscle nor lymphoid tissue: striated muscle.* Striated muscle is apparently unresponsive to the factor released during antigen-antibody interaction. Moreover, because of the relative lack of macrophages and lymphoid elements, injection into this tissue precludes the attainment of levels of antibody sensitization in other organs comparable, for example, to that after intracutaneous injection. This may be due to a partial destruction of antigen before sufficient amounts escape to antibody-forming centers elsewhere and, in part, to a slow release of antigen from the sequestered area in the muscle.

As will be seen, the above remarks are applicable to the discussion that follows. It is helpful to state here that, whereas other explanations for anaphylactic or transplantation reactions have invoked "tissue antibodies," we consider the mechanism to be nothing more mysterious than the reaction of the lymphocytes and other antibody-containing cells present in the respective tissues with specific antigen.

### *Transplantation*

Having presented the background in some detail above, I shall consider this topic only briefly. Transplantation "takes" or rejections depend, among other elements, upon the antigenic potency of the graft and the presence or absence of macrophages and lymphoid elements in the graft and at the locus of the transplant. Organs such as the adrenal and ovary are relatively nonantigenic, when placed in a site such as the seminal vesicle, which is poor in macrophages and lymphoidal elements, such grafts are sustained for some time. An organ such as the testis, when grafted to the seminal vesicle, is also maintained partly because of the same reason and, in part, because the antigenic portion of the graft, namely, the spermatogenic tissue, is destroyed by the higher abdominal temperature, leaving the nonantigenic portion.

Further remarks relating the concept to problems in transplantation are superfluous in view of the material presented by Medawar,[25] Lawrence,[26] Rapaport and Converse,[27] and Favour.[28] However, I am not yet convinced that the tuberculin type of hypersensitivity is the one to which transplantation reactions are most closely related.

With regard to acquired tolerance, I concur in the views expressed by Billingham[29] to the effect that antigen presented before initiation of antibody production (in ontogeny) or before reinitiation of antibody synthesis (that is, after adequate lymphocytolytic treatment, X irradiation, cortisone) is accepted as "native" material by the host. With less than adequate lymphoid depletion, response to antigens or homografts returns to the degree that antibody-forming tissue regenerates.

*Other Induced or Acquired Hypersensitive States in Which Organ-Specific or Cell-Specific Damage is Produced*

Experimentally, as mentioned before, a single injection of homologous sperm in adjuvant is sufficient to cause aspermatogenesis in guinea pigs Similar situations obtain in such conditions as uveitis,[22] neuritis,[23] and encephalomyelitis,[24] among others In order to apply the concept as described above, it is necessary to recall that these conditions are induced with relative success only when adjuvant is employed and that a factor in adjuvant that facilitates the reaction is contributed by bacteria Thus, upon injecting the antigen in adjuvant intracutaneously, the antigen is in effect incubated in a depot of oil in the skin During this incubation a great number of white blood cells, including macrophages and lymphocytes, is called to the area (Freund[30]) The number of the cells elicited by this method far exceeds that prompted by a saline vehicle Consequently, the great number of macrophages can account for a greater uptake of antigen at the site This may be one factor that is responsible for a heightened antibody formation, because it accordingly means that a greater number of antigenic units can be brought to the other organs of the body Another factor that can promote antigen pick-up by macrophages may be that the oil in which the antigen is present facilitates penetration of the antigen into the macrophages Moreover, the presence of bacteria and, more precisely, the polysaccharide therefrom, may simulate an infectious condition, in which case the defense mechanism of the host is mobilized to a greater degree than otherwise During the incubation period the antigen and polysaccharide might complex to form a more potent antigen These factors—intracutaneous injection, oil vehicle, and bacterial component-antigen complex—must be considered in the host response In any event, the antigen is transported throughout the body, and the organs that have depots of lymphoid tissue are stimulated to synthesize antibody The responses of many of the organs already have been considered

In such organs as the testes the possibility exists that the antigen brought to the testis reacts with lymphocytes in that organ Release of a toxic factor could lead to destruction The destruction can be self perpetuating because of the presence of endogenous antigen in the testis that can react with lymphocytes as these are brought to the organ via the circulation and draining lymph nodes As in the case of anaphylaxis, the more antibody cells that are brought in contact with the specific antigen present in the germinal cells, the greater the number of interactions that will occur Consequently, the reaction may go to completion and all antigen-bearing spermatogenic cells will be eliminated

The concept is applicable to the diseases mentioned previously and to other isoallergic syndromes It can also serve to explain how these disorders may occur spontaneously, as a result of such factors as damage or infection, an initial release of antigen from the organ stimulates autoantibody production against that organ It must not be overlooked that the isoallergic disorders are invariably associated with infections (see, for example, Woods[31] and Wintrobe,[32] relating respectively to uveitis and thrombocytopenia) Accordingly, the observations regarding the contribution of the bacterial component of adjuvant

assume great significance When the hypothesis is applied the following picture emerges during bacterial infection, damage to any antigenic organ with concomitant release of antigen permits complexing of antigen with the bacterial component The complex now becomes a unit against which antibody response is exacerbated because the antigen-lipopolysaccharide complex (1) represents a more formidable agent than the antigen alone, and (2) because the lipopolysaccharide component may facilitate penetration of the cells in the organ

It can be seen from the above presentation that the basic phenomena associated with a wide variety of immunological states are considered to be fundamentally similar to those involved in transplantation "takes" or rejects The proceedings of the Second Tissue Homotransplantation Conference[33] reveal that such thoughts are not alien to others in the field The advantage of the detailed presentation is that it permits experimental analyses of each postulate For example, now that it has been demonstrated that gamma-globulin is formed in the germinal centers of lymphatic nodules,[34] it should be possible to detect the synthesis of antibody in the lymphoid tissue of such organs as the ileum Also, if the findings are correct relative to the release of a factor (not histamine) that, during organ anaphylaxis, stimulates smooth muscle to contract *in vitro*, it should be possible to isolate and identify the factor

REFERENCES


1 Katsh, S , A S Gordon & H A Charipper 1947 Androgenic action of adrenal cortical transplants to the seminal vesicle of rats Anat Record **99** 105

2 Katsh, S , A S Gordon & H A Charipper 1948 The andromimetic activity of adrenal cortical transplants to the seminal vesicle of the adult rat Anat Record **101** 47

3 Katsh, S 1949 Androgenic activity of ovarian transplants to the seminal vesicle of the castrated adult male rat Anat Record **105** 40

4 Katsh, S 1950 Testicular and crystalline hormone implants in the rat seminal vesicle Federation Proc **9.** 69

5 Katsh, S 1950 The androgenic activity of ovarian transplants to the seminal vesicle of the castrated adult male rat Endocrinology **47** 370

6 Smith, A U 1949 Some antigenic properties of mammalian spermatozoa Proc Roy Soc London **B136** 46

7 Katsh, S 1957 *In vitro* demonstration of uterine anaphylaxis in guinea pigs sensitized with homologous testis or sperm Nature **180** 1047

8 Katsh, S & A Edelmann 1951 Adrenal cortical requirements of adrenalectomized, x-irradiated rats Federation Proc **10** 73

9 Katsh, S & E Kosarich 1952 The reproductive structures of the biotin-deficient male rat Anat Record **113** 394

10 Katsh, S , G F Katsh & P Osher 1954 Adrenal, pituitary and urinary ascorbic acid levels in rats subjected to hypothermic environment Am J Physiol **178** 457

11 Katsh, S , E Kosarich & J Alpern 1955 Effect of biotin deficiency on body and organ growth and accessory reproductive structures in male rats Growth **19.** 45

12 Katsh, S , G F Katsh & P Osher 1955 Organs of rats exposed to cold Anat Record **121** 402

13 Kennedy, W P 1924 The production of spermatotoxins Quart J Exptl Physiol **14·** 279

14 Freund, J , M M Lipton & G E Thompson 1953 Aspermatogenesis in the guinea pig induced by testicular tissue and adjuvants J Exptl Med **97.** 711

15 Katsh, S & D W Bishop The effects of homologous testicular and brain and heterologous testicular homogenates combined with adjuvant upon the testes of guinea pigs J Embryol Exptl Morphol In press

16 Katsh, S & D W Bishop Unpublished data

17 KATSH, S 1958 Demonstration *in vitro* of anaphylactoid response of the uterus and ileum of guinea pigs injected with testis or sperm J Exptl Med **107** 95
18 KATSH, S Unpublished observations
19 FREUND, J , G E THOMPSON & M M LIPTON 1955 Aspermatogenesis, anaphylaxis, and cutaneous sensitization induced in the guinea pig by homologous testicular extract J Exptl Med **101.** 591
20 KATSH, S Sensitivity of the ileum and the uterus and insensitivity of the vas deferens and seminal vesicle of sensitized guinea pigs to antigen *in vitro* To be published
21 MEDAWAR, P B 1948 Immunity to homologous grafted skin III The fate of skin homografts transplanted to the brain, to subcutaneous tissue and to the anterior chamber of the eye Brit J Exptl Pathol **29** 58
22 COLLINS, R C 1949 Experimental studies on sympathetic ophthalmia Am J Ophthalmol **32.** 1697
23 WAKSMAN, B H & R D ADAMS 1954 Experimental allergic neuritis produced in rabbits with nerve and adjuvants Federation Proc **13** 516
24 LUMSDEN, C E 1949 Experimental allergic encephalomyelitis Brain **72** 517
25 MEDAWAR, P B 1954 General problems of immunity Ciba Symposium on Transplantation Little, Brown & Co Boston, Mass
26 LAWRENCE, H S 1957 Similarities between homograft rejection and tuberculin-type allergy a review of recent experimental findings Ann N Y Acad Sci **64**(5)· 826
27 RAPAPORT, F T & J M CONVERSE 1957 Observations on immunological manifestations of the homograft rejection phenomenon in man the recall flare Ann N Y Acad Sci **64**(5): 836
28 FAVOUR, C B 1957 *In vitro* studies on cell injury in the tuberculin-type reaction implications in homotransplantation Ann N Y Acad Sci **64**(5): 842
29 BILLINGHAM, R E 1957 Studies on epidermal cell suspensions, with particular reference to problems of transplantation immunity Ann N Y Acad Sci **64**(5)· 799
30 FREUND, J 1957 Sensitization with organ specific antigens and the mechanism of enhancement of the immune responses J Allergy **28** 18
31 WOODS, A C 1956 Endogenous Uveitis Williams & Wilkins Baltimore, Md
32 WINTROBE, M 1956 Clinical Hematology 838 4th ed Lea and Febiger Philadelphia, Pa
33 ANNALS OF THE NEW YORK ACADEMY OF SCIENCES 1957 Second Tissue Homotransplantation Conference J M Converse, Ed **64**(5): 735–1073
34 ORTEGA, L G & R C MELLORS 1957 Cellular sites of formation of gamma globulin J Exptl Med **106.** 627

# SOME REACTIONS OF H-2 ANTIBODIES *IN VITRO* AND *IN VIVO*

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The importance of the histocompatibility-2 (H-2) system of antigens in homograft reactions in mice is now universally recognized This is not the place to discuss the genetic basis of this system, but a few general comments may be pertinent The H-2 antigens are widely distributed among the normal tissues of mice The components that have been described until now have been detected by hemagglutination techniques [1-5] However, there are certainly a number of very important components that are either absent from the erythrocytes or present upon them in very small quantity, and some of these have been identified by cytotoxic techniques These include a very potent antigen in H-2k and an allelomorph of antigen K in H-2b called $K^b$ It is therefore preferable to refer to the antibodies as H-2 antibodies rather than as hemagglutinins

Until recently doubts have been expressed as to the importance of H-2 antibodies in homograft reactions Three main objections have been put forward

(1) It appeared at first as if the hemagglutinating antibodies were very capricious in their appearance and transient in the circulations Since the adoption of the human serum dextran technique[6] this objection has been shown to be groundless Antibodies are regularly formed following homograft rejection The time during which they persist is somewhat variable but, following a strong primary stimulus, it varies between 3 and 13 months

(2) Mitchison and Dube[7] published evidence suggesting that antibody was formed relatively late in the homograft reaction and was therefore not important This finding is contrary to that of Kaliss[8] and to our own data There can be little doubt that the findings of Mitchison and Dube were due to the dextran they used We made direct comparison of their dextran with our own Not infrequently our dextran would give titers of over 1000, while theirs gave negative results However, we did find that the time of appearance of antibodies was extremely irregular Sometimes they could be detected on the fifth day, while on other occasions they might not be recognizable until much later It seemed possible that the antibody formed early might sometimes be incomplete, and we have done a number of experiments to test this hypothesis The method is somewhat complicated, and a brief description of it has been published elsewhere [9] Fuller details will appear shortly We studied an A-strain mammary carcinoma (AMT 1), a C57BL leukosis (E L 4), and the C3H ascites sarcoma B P 8 The results with all of these are in complete agreement with those of Kaliss[8] with the A-strain sarcoma 1 (Sa 1) Antibody may sometimes be detected on the third day and with regularity on the fourth day Visible signs of the homograft reaction have not been apparent until the fifth day with any of these tumors, and the appearance of antibody precedes them

(3) A great deal of stress has been laid upon the alleged lack of cytotoxic activity of H-2 antibodies I have never been impressed by these arguments A number of instances are known in which antibodies not lethal *in vitro* are highly effective *in vivo*, the Rh antibodies are an example However, this point would not have arisen if the H-2 system had been discovered in rats, since the serum of these animals has good complementary activity Mouse serum shows complementary activity *in vivo* since Amos (personal communication) has shown that homologous leukemic cells may be destroyed in diffusion chambers, and Algire *et al* [10] have found the same with HeLa cells However, such activity is rarely to be shown *in vitro* Ritz[11] thought mouse serum lacked end piece, while Brown[12] claimed that C′2 was lacking McGhee[13] found it possible to demonstrate complementing of antibodies against sheep red cells by the employment of very light cell suspensions My co-workers and I have performed a large number of experiments on this point In our experience it is very rarely possible to demonstrate complementary activity with mouse sera, and often such sera are strongly anticomplementary However, occasionally complementary activity is quite strong in hemolytic systems, and we have demonstrated cytotoxic activity with isoantibodies against B P 8 and E.L 4 The reasons for this peculiar property of mouse sera are unknown, but it is obvious that complement from some other species must be used to test the cytotoxic activity of mouse antibodies We found that either guinea pig or rat complement was suitable [14] We used the entry of eosin or trypan blue into the cell as index of cell death Here I propose to summarize the results originally reported, together with some new data Cells from normal spleen, lymph nodes, and bone marrow* are highly susceptible We have now tested three C57BL leukoses and two BALB/c leukoses All may be classified as susceptible, although there appear to be differences in sensitivity between them that require more precise investigation The ascites sarcoma B P 8 is of a lower order of susceptibility Under optimal conditions from 25 to 30 per cent of cells remain apparently undamaged The cells of the A-strain sarcoma 1 are probably completely resistant *in vitro* Our results with leukotic cells are very similar to those obtained by Schrek and Preston with a rat leukemia,[15, 16] and our results with spleen and lymph node cells have been confirmed by Klein *et al* [17]

I have called attention to the fact that there are at least three histological varieties of homograft response in mice [5, 9] In Type 1 (the classic type) the graft is supplied with a fibrovascular stroma by the host Graft destruction is intimately associated with infiltration by lymphocytes and plasma cells The early work of Russell[18] showed that in actively immunized mice no stroma was provided, and the growth persisted as a small, thin-walled cyst for some days

The Type 2 response has been found with ascites sarcomata We had an A-strain sarcoma that we converted to an ascites tumor The solid tumor gave a typical Type 1 reaction, while the converted tumor gave a Type 2 response

* Further experience has shown that most samples of bone marrow contain about 40 per cent of cells that appear undamaged *in vitro* However, the lethal effects of antibody on homologous marrow in irradiated mice has been confirmed

B P 8 and Sa 1 each gave a Type 2 response If grown under the skin or in the gastrocnemius muscle, ascites sarcomata do not evoke a fibrovascular stromal reaction As the tumor grows, host capillaries open up and the tumor virtually vascularizes itself When growing as a homograft in mouse strains A, BALB/c, or C57BL the picture is much the same in each It is also essentially similar both as a subcutaneous or intramuscular homograft except that the reactions are considerably more rapid at the latter site The subcutaneous graft is much the better for studying the vascular reactions FIGURE 1*a* shows B P 8 as 7-day old subcutaneous homograft in BALB/c Only one giant cell is shown, and the frequency of these bodies is somewhat variable The essential feature of the Type 2 reaction is a local proliferation of histiocytes that invade the tumor from the periphery and surround the malignant cells, which then die With subcutaneous homografts the first sign of the histiocytic reaction may be seen to begin on the fifth day and to become functional on the seventh or eighth day FIGURE 1*b* shows a well-developed histiocytic response on the ninth day On intramuscular implantation the reactions seem to occur about 48 hours sooner than in subcutaneous implantation, and the host appears more strongly sensitized The histiocytes do not appear to be much damaged by contact with the target cells in subcutaneous grafts, although they ultimately become engorged with cellular debris and disintegrate However, with intramuscular grafts, both histiocytes and target cells may disintegrate shortly after contact

With cell doses of up to 4 million cells, regression of a subcutaneous graft of B P 8 is usually complete at about 14 days With doses 3 to 4 times higher, the graft may persist for more than 3 weeks In such cases one may often see perivascular areas of necrosis from the ninth day onwards These very likely represent a direct action of antibody I have never seen plasma cells in the region of a Type 2 homograft Lymphocytes are sometimes present in considerable numbers near the graft, but they do not invade it

The secondary response is essentially a violent recapitulation of the primary response, except that circulating monocytes seem to play a part Histiocytes and target cells are both rapidly killed Sometimes no traces of living cells are to be found after the third day Occasionally the cellular response seems to die away on the fourth day The remainder of the graft persists in an indolent state, with some peripheral cells dying without cellular invasion, perhaps killed by antibody The process is finally brought to a close about the seventh day when infarction occurs Medawar[19] was the first to draw attention to the importance of vascular occlusion with skin homografts, while Algire[20] demonstrated the phenomenon with a mammary tumor homograft

Potter and Findlay[21] were the first to demonstrate the Type 3 reaction, although they did not so name it The conspicuous feature of leukotic homografts is exudation of plasma This may be visible as a slight edema on the fifth day with subcutaneous homografts of our C57BL leukosis E.L 4 By the sixth or seventh day the whole flank is intensely edematous FIGURES 2*a* and *b* show the exudative reaction of sixth- and seventh-day homografts of E L 4 in BALB/c mice At first the reaction is confined to the deepest part of the graft, later, however, areas of necrosis occur throughout Proliferation of

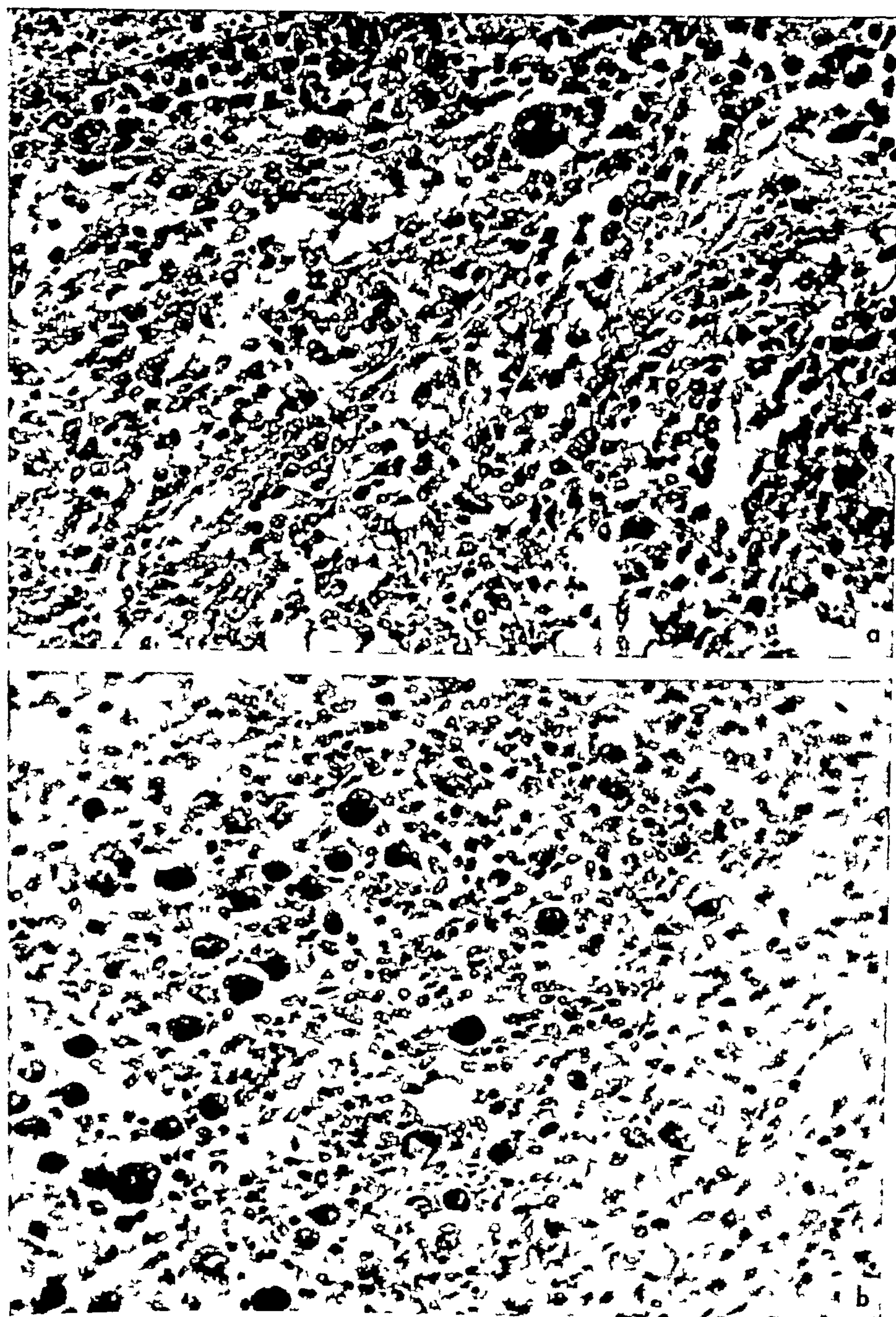


FIGURE 1 (*a*) B P 8 ascites sarcoma growing as a seventh-day homograft in BALB/c mouse Hematoxylin and eosin ×90 (*b*) The Type 2 homograft response B P 8 ascites sarcoma ninth-day homograft in BALB/c mouse Well-developed histiocytic response ×90

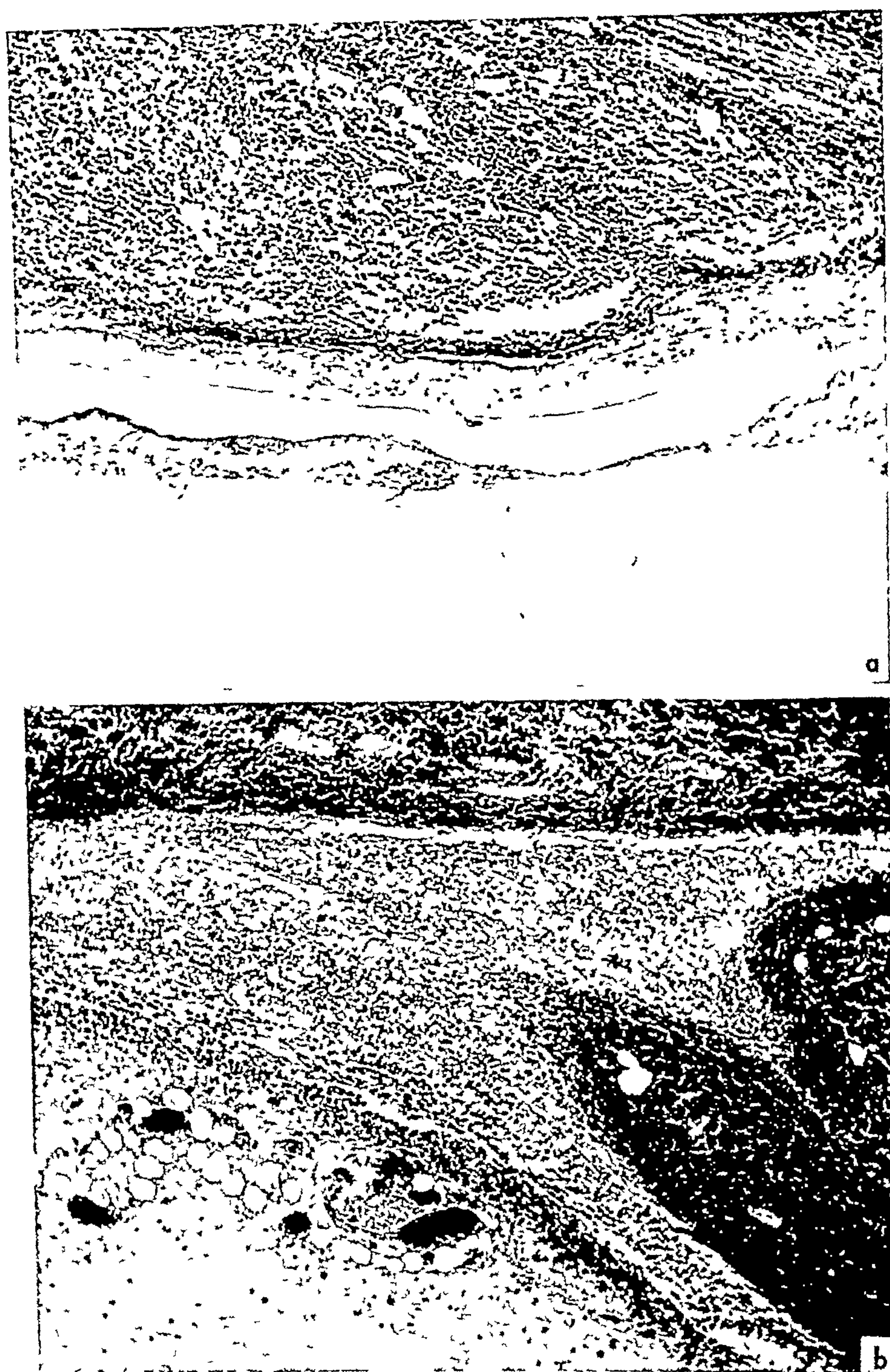



FIGURE 2 (*a*) Early Type 3 homograft reaction E L 4 leukosis growing as sixth-day homograft in BALB/c mouse Note exudation in lower part of the graft ×90 (*b*) Well-developed Type 3 homograft response E L 4 leukosis as seventh-day homograft in BALB/c mouse Note very extensive exudation and extensive destruction of malignant cells ×90

host cells in the graft bed does not being until the eighth or ninth day Invasion of the graft by histiocytes may be seen on the tenth day These act very largely as scavengers, but they are quite capable of destroying the leukotic cells We have not studied the secondary response, but it seems to be a very rapid recapitulation of the primary response

The first demonstration of cytotoxic or cytostatic effects of antibody *in vivo* was done with isografts [22] In this case antibody produced in C57BL mice and A-strain leukemic cells were mixed *in vitro* and then inoculated subcutaneously into A-strain mice Usually growth was temporarily arrested, but occasionally it was accelerated, an A-strain sarcoma was strongly accelerated This technique we now call the "neutralization" technique, and it has been successfully used by others Billingham and Sparrow[23] were able to demonstrate a cytostatic effect in this way using homografts of dissociated epidermal cells in rabbits If target cell and antibody meet for the first time *in vivo* we now refer to a "passive-immunity technique" In general, neutralization and passive immunity give similar results, but the former is the more sensitive

The successful demonstration of passive immunity to leukemic cells has already been reported [24, 25] Only a few points need stressing here The effectiveness of antibody in the suppression of homografts is what might have been anticipated from results obtained *in vitro* (in point of fact passive immunization was observed first) However, we also found an antibody that will give some protection in the isologous strain We called the corresponding antibody anti-X Unfortunately anti-X gives no cytotoxic effect *in vitro* Nathan Kaliss and I performed a number of experiments on the effects of serum dosage on the behavior of homografts of the C57BL leukosis E L 4 , the C3H sarcoma B P 8, and the A-strain Sa 1, full details of which will be published All three gave different results E L 4 gave the type of result that might have been anticipated with bacteria or viruses With small doses one got no effect, some protection with intermediate doses and, finally, complete protection We had previously reported[24] that passively protected mice were completely susceptible to a second challenge Both of these observations differ from those made with B P 8

E A Boyse and I are now engaged in a series of experiments to test the activity of antibodies on bone marrow and spleen A-strain mice that have received a fatal dose of X rays (900 r) are given good temporary protection by 3 million C3H marrow cells Prior administration of 0 2 ml of a potent serum produced in A-strain mice against B P 8 completely abolished the protective effect Positive results have also been obtained with spleen, but the results are less clear-cut, since we got very poor protection of A-strain mice with C3H spleen

Nathan Kaliss and I shall describe details of "passive immunity" experiments by B P 8 elsewhere A number of variables influence the result With high doses of serum one may get almost complete suppression of the growth With lower doses one occasionally gets an initial inhibition followed by permanent homografts On other occasions the tumors may grow at about the same rate as in controls, but they regress more slowly or persist Suppression of the growth and an initial delay can occur with intramuscular homografts, but

enhancement is more easily demonstrated Sometimes the growth rate is so greatly accelerated at this site that the host seems to be overwhelmed

There have been suggestions that enhancement might be due to the fact that antigen combined with antibody was an inefficient stimulus[15, 26, 27] If this is the case it would appear that the onset of the homograft response should be delayed, if not suppressed I have done a number of experiments to test this hypothesis, a few of them have been described[9] In most of these, three groups of mice were used a control group given no serum and two groups given the same dose of serum The controls and members of the first serum group were sacrificed at different times, while the second serum group was left alive to determine the fate of the tumor

In controls the first macroscopic sign of the homograft reaction response is a general vasodilation and edema that is usually apparent on the fifth day We refer to this as "the flare" Shortly afterward an area of intense congestion, referred to as the halo, appears around the graft The flare and halo usually fade about the ninth or tenth day, even though the graft may contain living cells for a considerably longer period

In some experiments the tumor was so strongly inhibited by antibody that it was scarcely palpable on the sixth day and the growths appeared almost completely necrotic, some no larger than those in controls at 24 hours However, some showed a well-developed flare Histological examination showed a small shell of living cells Even where there was no flare or halo there was a very marked histiocytic response That shown in FIGURE 3 is far better devel-

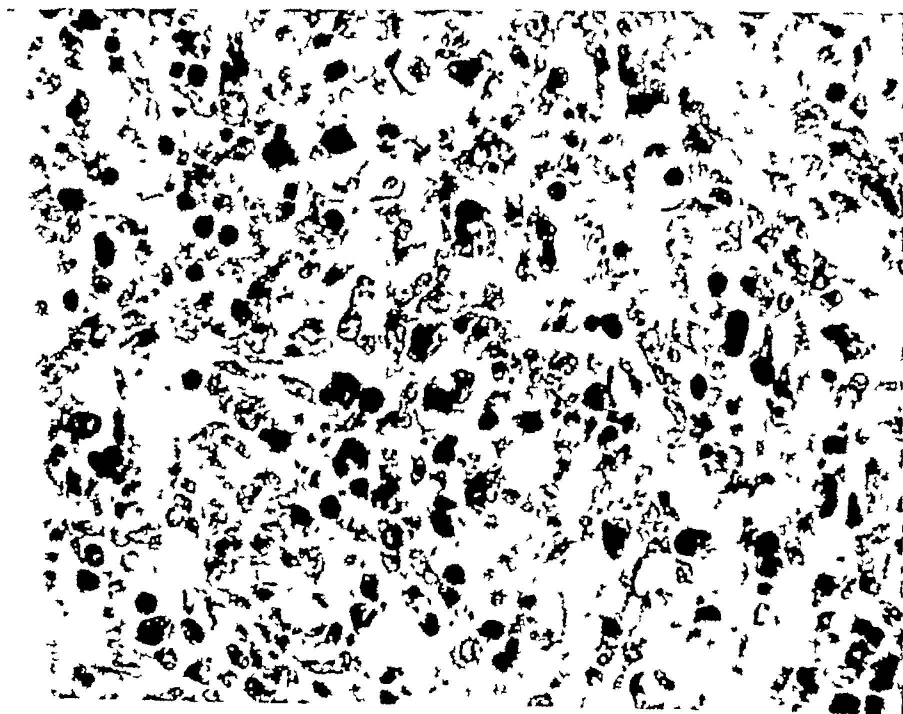


FIGURE 3 B P 8 ascites sarcoma in A-strain mouse given 0 2 ml Strain A anti-B P 8 two hours prior to receiving B P 8 The growth had not increased in size 48 hours postinoculation Nevertheless, there is a very marked histiocytic response on the sixth day, it is far better developed than in controls ×90

oped than in a 6-day control In this experiment the second serum group, rechallenged 1 month after the first inoculation, showed evidence of active immunization—a result that differs markedly from that obtained with E L 4

Good enhancement is difficult to obtain in A-strain mice under any conditions but fairly easy in BALB/c mice If delay in the onset of the homograft reaction is of vital importance in enhancement this should be particularly obvious in those cases in which strong initial inhibition is followed by a permanent growth On the fifth day such tumors look exactly like the permanently suppressed growths, however, they often show a very advanced flare and halo The histological appearance is very interesting FIGURE 4*a* shows that a great deal of the tumor is reduced to a cyst with a wall one cell thick, and there are signs of a cellular reaction in the graft bed This shows a remarkable resemblance to the illustration of the old carcinoma 27 illustrated as a fourth-day graft in an actively immunized mouse in FIGURE 4*b* taken from Russell's classic paper published in 1908 FIGURE 5*a* taken from the same paper, shows the Jensen carcinoma as a sixth-day graft in an immune mouse

Similarly, there are several mitotic figures in the fifth-day graft of B P 8 shown in FIGURE 5*b* However, there is considerable infiltration by host cells and, in some parts, a good histiocytic response may be shown This has never been seen so early with subcutaneous controls By the seventh day the inhibitory effect of antibody was beginning to wane, and the growths were well established by the ninth day in spite of a persistant histiocytic response

All the tumors in the second serum group persisted, and some metastasized In other cases in which the control and experimental tumors are initially of about the same size there is no visible difference in the homograft response macroscopically or microscopically In the experimental group the histiocytic reaction is obviously functional for a time, but it ultimately fades away on the tenth day or later The only time in which the homograft reaction is initially suppressed is if the growth rate is violently stimulated from the beginning, by comparison with control growth

Sarcoma 1 gives a very different picture, since growth is enhanced over a very wide range of antibody concentration and no indication of inhibition was observed

Borges (quoted by Kaliss[28]) noted histiocytic proliferation in homografts of Sa 1, but he did not follow the process to completion, without prior knowledge gained from other tumors it would be extremely difficult to do so The distinctive cells of B P 8 are easy to differentiate from histiocytes even when dead, while those of Sa 1 are not, at least in section However, careful search will show that the histiocytic responses are essentially the same in both In the case of B P 8 the histiocytes are not immediately damaged on contact, but seem to become engorged with cellular debris and then to disintegrate In the case of Sa 1, disintegration seems to be very rapid, and one usually sees an area of necrosis separating histiocytes and tumor There is a suggestion that the violence of this reaction is somewhat diminished late in the homograft reaction when the titer of antibodies is rising rapidly

In the case of serum-treated animals our observations agree with those of Borges with actively enhanced mice. There is no sign of any delay in the time




FIGURE 4 (*a*) Fifth-day homograft of B P 8 ascites sarcoma in BALB/c mouse given 0 5 ml BALB/c anti-B P 8 two hours prior to inoculation of tumor The growth was very largely inhibited and, for the most part, it consisted of a thin-walled cyst with a cellular reaction surrounding it, as shown in the figure The growths ultimately recovered from the inhibition by antibody and finally killed their hosts ×180



FIGURE 5 (*a*) Drawing by Russell[18] of another carcinoma in an actively immunized mouse This shows mitotic arrest (*b*) Mitotic arrest brought about in BALB/c mouse by 0 5 ml of BALB/c anti-B P 8 ascites sarcoma given 2 hours prior to inoculation of tumor Same growth as illustrated in FIGURE 4*a* The result of this partial arrest is to increase the number of heteroploid cells in the tumor ×180

of onset of the homograft response In our experiments the explosive histiocytic reaction was first observed on the seventh day in controls In serum-treated animals it failed to mature In its place there was a violent increase in mitotic activity in the tumor

It does not seem permissible to discuss enhanced homograft survival in isola-

tion from homograft reactions as a whole At least insofar as H-2 antagonism is concerned we must now abandon the idea that H-2 antibodies are nonexistent or unimportant The results of passive administration of antibody depend to a large extent upon the composition of the target-cell population At one extreme we have the highly susceptible E L 4 So far the only effect observed has been inhibition At the other extreme we have Sa 1 Here the only direct effect of antibody to be observed upon the neoplastic cells has been the stimulation of mitosis Enhancement is very easy to obtain with a wide range of antibody concentration One suspects that B P 8 is more representative of the bulk of neoplastic homografts A very important variable is the concentration of serum With certain serum doses very little may happen, while striking results may be obtained in the same experiment with larger or smaller doses One wonders how many interesting observations have been missed owing to this type of complication

When we use serum doses capable of causing marked inhibition of B P 8, sensitization of the host may be accelerated up to a point Under optimal conditions the tumor is so efficiently arrested by antibody that the cellular defenses of the host become fully mobilized before the tumor can recover and the animal is actively immunized In effect, we have produced a subclinical attack of the disease

If conditions are made more favorable for the tumor it may recover although the host has been sensitized to some extent We know that B P 8 is a heterogeneous population of cells so far as sensitivity to antibody is concerned It seems reasonable to suppose that selection will have increased the proportion of antibody-resistant cells, however, this need not be the *only* effect We have shown that antibody may have a colchicinelike effect on the mitotic apparatus Sometimes the cells die in mitosis, at other times they may complete abnormal mitosis giving rise to large heteroploid cells as shown in FIGURE 6*a* It would appear that such cells may be very resistant to the action of histiocytes since they often engulf large numbers of them as shown in FIGURE 6*b*

If conditions are sufficiently favorable to the tumor we may get simple stimulation of mitosis without any sign of damage to the cells We appear to have two satisfactory factors to account for enhanced growth of B P 8 If antibody is present in subeffective amounts we may produce a highly resistant tumor, or the tumor may be so violently stimulated by subeffective doses of antibody that the host is overwhelmed Clearly, both factors could act synergistically

It is very likely that both factors play their parts under certain circumstances, but they do not seem to give a really satisfactory account of all phenomena They can hardly account for the prolongation of ovarian[29] or skin homografts[26] produced by treatments similar to those effective with tumors It is possible that enhancement of normal homografts is brought about by a different mechanism, indeed it has yet to be shown that passively administered antibody can enhance normal homografts This is not a very satisfying argument One may doubt whether the factors mentioned in connection with B P 8 are operative in quite the same way with Sa 1 It seems unlikely that antibody should have any selective effect on Sa 1, which is resistant already The stimulation of

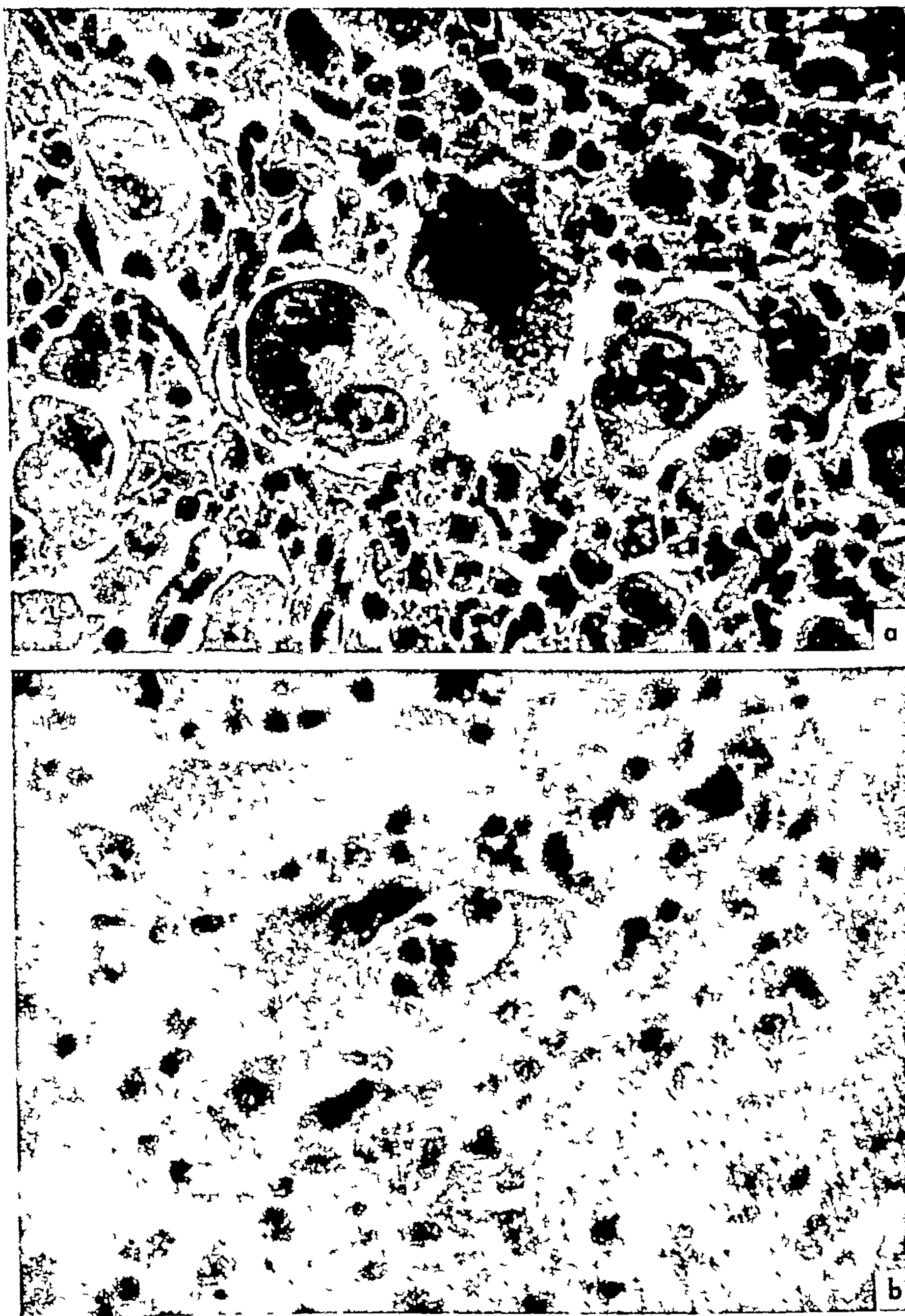



FIGURE 6 (*a*) Large heteroploid cells in homograft of B P 8 ascites sarcoma in BALB/c mouse pretreated with 0 5 ml of serum There is a very precocious infiltration by histiocytes as compared with controls, however, they do not appear to be fully functional at this time ×450 (*b*) A seventh-day homograft of B P 8 ascites sarcoma in BALB/c mouse receiving 0 5 ml. of serum The growth is beginning to recover from the inhibitory effects of antibody Although the histiocytic response is now apparently functioning well, some cells appear to be resistant to histiocytic action The large heteroploid cell shown has engulfed many histiocytes ×450

mitosis observable on the seventh day is certainly detrimental to the host, but it does not seem very satisfactory as an explanation of the persistence of the growth

If we exclude overwhelmingly stimulated growths, the one factor that is common to enhancement of both B P 8 and Sa 1 is the apparent normal commencement of a homograft response that fades prematurely An identical appearance may be seen with the so-called nonspecific tumors that grow as homografts without any pretreatment An assumption here has been that the tumor itself has paralyzed the host in some way This may be so, but there is an interesting possibility that antibody may play an important part in the fade-out

During the evolution of the homograft reaction we may observe an increasing sensitivity in the host's cells as revealed by phenomena such as the flard and halo and an increasing titer of antibody The vascular reactions and other cellular responses are presumably elicited by antigenic material derived from the graft Some of this material may combine with antibody and thus reduce the amount reaching the sensitized cells Usually this is probably unimportant, since the level of sensitivity appears to rise as the titer of antibody rises We have seen that the time of onset of the homograft reaction is not necessarily delayed if enhancement is to occur, and the host will produce antibody itself, therefore we may be establishing an amount of antibody that is excessive for the degree of cellular sensitivity attained at a given time The violence of the histiocytic reaction against Sa 1 suggests that a very high level of sensitivity is essential if the graft is to be destroyed Antibody is apparently entirely innocuous to this growth and, apart from stimulating mitosis, it may interfere with the violence of the cellular response by reducing the amount of antigen reaching the histiocytes I have referred to this as the anergic hypothesis [5]

## *Summary and Conclusions*

(1) Isoantibodies reacting with H-2 antigens are regularly present from the third or fourth day in mice receiving homografts They may persist for as much as a year following a single challenge

(2) Three histologically distinct types of homograft response are described in the text

(3) In the presence of complement, such antibodies are cytotoxic *in vitro* to normal cells from spleen, lymph nodes, and bone marrow * Cells from 5 cases of leukemia were found to be susceptible The C3H ascites sarcoma B P 8 contains about 25 per cent of cells that are resistant to cytotoxic action The cells of the A-strain sarcoma 1 appear to be completely resistant

(4) In fatally irradiated mice the protective action of homologous marrow may be abolished by prior injection of H-2 antibodies Destruction of splenic cells was also demonstrated

(5) Homografts of leukemic cells may be inhibited by antibodies Passive immunity to isografts has also been demonstrated The antigens and anti-

* See footnote, page 708

bodies concerned in this type of reaction have been termed X and anti-X, respectively Animals protected by H-2 antibodies or by anti-X are fully susceptible to further challenge

(6) Large doses of antibody may cause permanent arrest of homografts of B P 8 Not all the cells are killed by antibody The survivors are destroyed by host histiocytes and the animals are actively immunized

(7) Smaller doses of antibody may cause temporary inhibition of B P 8 The tumor that finally emerges frequently kills the host in spite of an early, well-developed homograft response

(8) The growth rate of B P 8 may be greatly accelerated by *small* doses of antibody if the homografts are intramuscular

(9) Sarcoma 1 shows enhancement with all doses of antibody studied

(10) Delay in the time of onset of the homograft response does not seem to be important in enhancement by antibody In the case of B P 8 the host may be overwhelmed by a greatly accelerated tumor Under other conditions immunoselection may be operative, and new polyploid cell types may be produced through interference with mitosis by sublethal doses of antibody

In the case of Sa 1, antibody does not delay the time of onset of the homograft response but appears to interfere with its proper functioning It is suggested that excess of antibody may desensitize by reducing the amount of antigen reaching the histiocytes

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*References*

1 GORER, P A 1937 The genetic and antigenic basis of tumour transplantation J Pathol Bacteriol 47 231–252
2 GORER, P A, S LYMAN & G D SNELL 1948 Studies on the genetic and antigenic basis of tumour transplantation Linkage between a histocompatibility gene and "fused" in mice Proc Roy Soc London **B135** 499–505
3 HOECKER, G, S COUNCE & P SMITH 1954 The antigens determined by the H-2 locus a rhesus-like system in the mouse Proc Natl Acad Sci **40** 1041–1051
4 AMOS, D B, P A GORER & Z B MIKULSKA 1955 An analysis of an antigenic system in the mouse (the H-2 system) Proc Roy Soc London **B144** 369–380
5 GORER, P A 1956 Some recent work on tumor immunity Advances in Cancer Research **4** 149–186
6 GORER, P A & Z B MIKULSKA 1954 The antibody response to tumor inoculation Improved methods of antibody detection Cancer Research **14.** 651–655
7 MITCHISON, N A & O L DUBE 1955 Studies on the immunological response to foreign tumor transplants in the mouse III The relation between haemagglutinating antibody and graft resistance in the normal mouse and mice pretreated with tissue preparations J Exptl Med **102** 179–197
8 KALISS, N 1957 The survival of homografts in mice pretreated with antisera to mouse tissue Ann N Y Acad Sci **64**(5) 977–990
9 GORER, P A Some varieties of homograft response Colloques Internat No 78 Centre National de la Recherche Scientifique Paris, France In press
10 ALGIRE, G H, WEAVER, J M & R T PREHN 1957 Studies on tissue homotransplantation in mice, using diffusion-chamber methods Ann N Y Acad Sci **64**(5). 1009–1013

11 Ritz, H 1911 Über Antikorperbildung und Anaphylaxie bei weissen Mausen Z Immunitatsforsch **9** 321–344

12 Brown, G C 1943 The complementary activity of mouse serum J Immunol **46:** 319–323

13 McGhee, R B 1952 Presence of complement in serum of the mouse Proc Soc Exptl Biol Med **80** 419–425

14 Gorer, P A & P O'Gorman 1956 The cytotoxic activity of isoantibodies in mice Transplantation Bull **3** 142–143

15 Schrek, R & F W Preston 1956 Toxicity of homologous immune serum to transplantable tumor Studies using phase microscopy and cinemicrography J Natl Cancer Inst **16** 1021–1045

16 Schrek, R & F W Preston 1957 Cytotoxicity of a homologous immune serum to a transplantable rat tumor by the method of unstained cell count Cancer Research **17** 102–107

17 Klein, E, G Klein & L Revesz 1957 Permanent modification (mutation) of a histocompatibility gene in a heterozygous tumor J Natl Cancer Inst **19** 95–114

18 Russell, B R G 1908 The nature of resistance to the inoculation of cancer 3rd Sci Rept Imp Cancer Research Fund · 341–358

19 Medawar, P B 1944 Behaviour and fate of skin autografts and homografts in rabbits J Anat **78** 176–199

20 Algire, G H 1954 Vascular reactions of normal and malignant tissues *in vivo* VII Observations on vascular reactions in destruction of tumor homografts J Natl Cancer Inst **15.** 483–491

21 Potter, J S & M D Findley 1935 Histological observations on resistance to transplantable leukaemia in immunised mice Proc Soc Exptl Biol Med **32**· 1338–1340

22 Gorer, P A 1942 The role of antibodies in immunity to transplanted leukaemia in mice J Pathol Bacteriol **54** 51–65

23 Billingham, R E & E M Sparrow 1954 Studies on the nature of immunity to homologous grafted skin, with special reference to the use of pure epidermal grafts J Exptl Biol **31** 16–39

24 Gorer, P A & D B Amos 1956 Passive immunity in mice against the C57BL leukosis E L 4 by means of iso-immune serum Cancer Research **16** 338–343

25 Amos, D B & E D Day 1957 Passive immunity against four mouse leukoses by means of isoimmune sera Ann N Y Acad Sci **64**(5) 851–858

26 Billingham, R E, L Brent & P B Medawar 1956 Enhancement in normal homografts with a note on its possible mechanism Transplantation Bull **3.** 84–85

27 Snell, G D 1956 The suspension of the enhancing effect in mice by the addition of donor lymph nodes to the tumor inoculum Transplantation Bull **3**· 83–84

28 Kaliss, N 1955 Induced alteration of the normal host-graft relationships in homotransplants of mouse tumors Ann N Y Acad Sci **59**(3) 385–391

29 Parkes, A S 1956 The attenuation of host reaction to ovarian homografts Nature **178** 1228

# OBSERVATIONS ON THE GENETIC RELATIONSHIPS AFFECTING THE TRANSPLANTABILITY OF SKIN IN INBRED MICE*

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Knowledge concerning the susceptibility and resistance to homologous tumor transplants is more extensive than that pertaining to the transplantability of normal tissues

In a recent review Law[1] formulated the following three generalities governing the growth of transplantable tumors transplantable tumors studied in mice (1) usually grow in all members of the strain of origin, (2) very likely do not grow progressively in unrelated strains of mice, and (3) grow in all of the $F_1$ progeny when one of the parents is from the strain of origin of the tumor One can postulate that normal tissues will demonstrate the same growth patterns as tumors Counce *et al*[2] showed that the determinants of resistance and susceptibility of normal and abnormal tissues may be similar However, the growth potential and the complexity of antigenic components in the transplant, as well as the influence of hormones and unknown factors, may confuse the picture sufficiently to warrant caution in comparing the observations of transplantability of tumors and normal tissues

In a series of experiments utilizing inbred strains of mice, some with known histocompatible genes, the authors studies the growth characteristics of full-thickness isologous and homologous skin grafts Full-thickness skin grafts 1 sq cm in diameter were taken from the donor animal and transferred to a patterned surgical interscapular defect in the recipient Fixation was done with interrupted 5-0 black silk sutures Full-thickness skin grafts, rather than split-thickness grafts, were used in all of our experiments because acceptance of the former graft requires more favorable local conditions for growth than the latter Infection, surgical technique, nutrition, and revascularization are some of the factors that are of importance for the viability of a skin graft The authors feel that observations on the viability of full-thickness skin grafts are, therefore, more valid because the conditions affecting acceptance are more stringent

Preliminary results were obtained using 4 inbred strains procured from John Bittner, University of Minnesota, Minneapolis, Minn , and bred in our laboratories In a previous report[3] dealing with these strains it was shown that hair color was a determining factor for the growth of homologous skin TABLE 1 includes the results of this experiment and extended studies The histocompatible genes at the H-2 locus are known for 3 of the strains $H^{-2k}$ for C3H and CBA, and $H^{-2b}$ for C57 As can be seen from these data, skin from C mice grew readily in C, CBA, and C57 strains and, to a lesser extent, in C3H mice In all instances homologous skin grafts were rejected by the C

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TABLE 1

TRANSPLANTABILITY OF SKIN GRAFTS AMONG VARIOUS INBRED STRAINS OF MICE

| Donor strain and hair color | Recipient strains | | | |
|---|---|---|---|---|
| | C | C3H | CBA | C57 |
| C (White) | 20/27* (74%) | 6/15 (40%) | 22/30 (73%) | 9/13 (69%) |
| C3H (Brown) | 0/9 (0%) | 13/18 (72%) | 7/18 (39%) | 0/19 (0%) |
| CBA (Brown) | 0/13 (0%) | 6/11 (55%) | 17/22 (77%) | 0/18 (0%) |
| C57 (Black) | 0/22 (0%) | 0/15 (0%) | 0/14 (0%) | 14/14 (100%) |

* Numerator = number of viable skin grafts 30 or more days after grafting, denominator = total number of recipients

strain Homologous skin was transferred successfully between the C3H and the CBA strains The other strains rejected transplants from these mice Homologous C57 skin was rejected in all 3 recipient strains From these results it can be seen that homologous skin from the white strain grew readily and that skin from the strain with a black coat color was rejected in each instance The acceptance of skin from the 2 mouse strains with a brown coat color was in an intermediate position If the histocompatible genes at the H-2 locus were of primary importance for acceptance of homologous skin grafts, one might expect that reciprocal transfer of homologous skin between the C3H and CBA mice would be successful However, the acceptance of C skin by C3H, CBA, and C57 strains is an indication of a similarity among all 4 strains which, as yet, cannot be explained by the existence of known histocompatible genes In addition, the rejection of homologous skin from the C3H and CBA and the acceptance from the C mice by the C57 strain could not be explained only by the presence of the $H^{-2b}$ allele unless it is assumed that this allele produces an environment that is not antagonistic to C skin Another possibility seems feasible, namely, the antigenicity of the pigments[4] involved may be more directly concerned with the acceptance or rejection of these homografts Thus, if it were assumed that the lack of pigment in the albino strain would eliminate the involvement of an antigenic pigment and that the differently pigmented coat colors of the other strains would serve as antigenic bases for rejection, it could be predicted that acceptance of homologous skin would occur only in these donor-recipient combinations C to C3H, CBA, and C57, C3H to CBA, and CBA to C3H

In a second experiment, only strains with known histocompatible genes at the H-2 locus were employed The results, shown in TABLE 2, demonstrate nearly complete rejection of homologous skin grafts Homografts were accepted in only 4 donor-recipient combinations and then in but a very small number of animals There is no obvious correlation between coat color and the fate of the homografts, nor does it appear that the histocompatible genes were responsible for the results Perhaps genetic and/or antigenic factors other than those known to be responsible for acceptance of tumors may be exerting an influence on the growth of homologous skin grafts

$F_1$, $F_2$, and backcross progeny of the strains presented in TABLE 2 are being

TABLE 2
TRANSPLANTABILITY OF SKIN GRAFTS AMONG MOUSE STRAINS WITH KNOWN HISTOCOMPATIBLE GENES

| Donor strains, histocompatible genes, and hair color | Recipient strains | | | | | |
|---|---|---|---|---|---|---|
| | C57BR/cd | C57BL/6 | BALB/c | DBA/1 | DBA/2 | A/Jax |
| C57BR/cd $H^{-2k}$ (Brown) | 6/9* (67%) | 0/10 (0%) | 0/10 (0%) | 0/10 (0%) | 0/22 (0%) | 0/10 (0%) |
| C57BL/6 $H^{-2b}$ (Black) | 0/10 (0%) | 7/10 (0%) | 0/10 (0%) | 0/10 (0%) | 0/10 (0%) | 0/10 (0%) |
| BALB/c $H^{-2d}$ (White) | 0/9 (0%) | 0/25 (0%) | 7/10 (70%) | 0/27 (0%) | 0/10 (0%) | 4/29 (14%) |
| DBA/1 $H^{-2q}$ (Brown) | 2/18 (11%) | 0/10 (0%) | 0/10 (0%) | 8/9 (88%) | 0/9 (0%) | 0/10 (0%) |
| DBA/2 $H^{-2d}$ (Brown) | 0/10 (0%) | 0/10 (0%) | 0/22 (0%) | 1/19 (5%) | 9/10 (90%) | 0/9 (0%) |
| A/Jax $H^{-2k}$ (White) | 2/17 (12%) | 0/10 (0%) | 0/10 (0%) | 0/10 (0%) | 0/9 (0%) | 7/10 (70%) |

* Numerator = number of viable skin grafts 30 or more days after grafting, denominator = number of recipients

studied in order to determine the distribution and the number of these histocompatible factors At the present time sufficient data have been accumulated to discuss the disposition of some of the $F_1$ progeny Studies evaluating the influence of sex and the distribution of coat color and antigenic components in the nuclei, mitochondria, and microsomes of the progeny are in progress

It was expected that the isologous skin grafts among the $F_1$ progeny would

TABLE 3
TRANSPLANTABILITY OF ISOLOGOUS SKIN GRAFTS AMONG $F_1$ PROGENY

| Parent strains | Proportions of viable transplants | |
|---|---|---|
| | Male donors and recipients | Female donors and recipients |
| A/Jax C57BL/6 | 5/12* 42% | 0/8 0% |
| C57BL/6 DBA/2 | 11/16 69% | Not done |
| BALB/c C57BL/6 | 17/36 47% | 4/21 19% |
| C57BL/6 DBA/1 | 8/20 40% | 2/12 17% |
| DBA/1 BALB/c | 16/20 80% | 4/11 36% |
| A/Jax BALB/c | 7/14 50% | 0/3 |
| DBA/2 A/Jax | 6/10 60% | 2/5 |
| DBA/2 BALB/c | 13/16 81% | 5/5 |
| DBA/1 C57BR/cd | 2/16 12% | 0/7 |

* Numerator = number of viable skin grafts at 30 or more days after grafting, denominator = total number of recipients

be accepted in the same proportion of the recipients as was observed among the parent strains (from 66 to 90 per cent of cases) However, the proportions of acceptances, as seen in TABLE 3, varied from 12 to 80 per cent among males and from 0 to 36 per cent among the females Percentages were used with small numbers only for comparative purposes, in some instances it seemed inadvisable to convert the proportions to percentages

Attempts were made to correlate the results obtained among the $F_1$ progeny with certain characteristics of the parent strains In no instance was any one parent strain responsible for either progeny with consistently high proportions of acceptances or progeny with consistently low proportions of rejections The same lack of correlation was evident when the coat colors of the parent strains were considered Finally, there were no consistent correlations between the fate of the isologous skin grafts and the histocompatible genotypes of the parent strains The preceding statements refer to the results obtained with male donors and recipients, a larger number of females would be necessary for extensive comparisons It appears, however, that among the $F_1$ progeny a larger proportion of isologous skin grafts was accepted among males than females These results indicate the possibility of sex-linked factors and the heterozygous nature of the parents with regard to histocompatibility when using full-thickness skin grafts Further studies are needed to determine the number and nature of these factors A more extensive study is in progress to determine the relationship of histocompatibility, distribution of coat colors, and distribution of antigenic components in various tissue fractions among $F_2$ and backcross progeny

In summary, the fate of homologous full-thickness skin grafts in certain inbred mice appears to be determined by a number of factors that may not be related to the histocompatible genes at the H-2 locus that are known to occur in these strains Antigenic components of the pigments characterizing the coat colors of the mice might have an influence on the acceptance and rejection of the homografts The variability of the fate of isologous skin grafts among the $F_1$ progeny indicates heterozygous parents with regard to factors that affect the acceptance of the transplants

### *References*

1 LAW, L W 1954 Genetic studies in experimental cancer Advances in Cancer Research 2 281–352

2 COUNCE, S , P SMITH, R BARTH & G D SNELL 1956 Strong and weak histocompatibility gene differences in mice and their role in the rejection of homografts of tumors and skin Ann Surg **144** 198–204

3 WERDER, A A & C A HARDIN 1955 Importance of hair color to successful skin transplantation A M A Arch Surg **70** 407–410

4 BILLINGHAM, R E & P B MEDAWAR 1950 Pigment spread in mammalian skin Serial propagation and immunity reactions Heredity **4**. 141–164

# THE NATURE OF TISSULAR ANTIGENS, WITH PARTICULAR REFERENCE TO AUTOSENSITIZATION AND TRANSPLANTATION IMMUNITY*

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## Introduction

Having studied autosensitization for ten years and homografts for five, we feel that it is time to define the possible relationships between them These two problems have long been considered unrelated, but we believe that they have many relationships as well as highly significant differences We also feel that consideration of these factors may throw some light on both of these problems The principal relationships are three. First, the two kinds of antigens (autoantigens and homograft antigens) involved in these phenomena are cellular or tissular antigens that give rise to intracellular or extracellular substances that act as "cytotoxic" antibodies Second, there are three sites from which one can undoubtedly extract autoantigens. the white substances of the brain, the lens, and the testes These are precisely the three sites at which it is possible to perform successful homografts the brain,[1] the anterior chamber of the eye, and the testes [2] This relationship must have some significance Third, the phenomenon of specific acquired tolerance concerns both of these kinds of antigens It involves the phenomenon by which homograft antigens cease to be antigenic in the experimentally prepared animals and also the phenomenon by which "autosubstances" that do not come into contact with the rest of the organism during embryonic life subsequently acquire the properties of autoantigens

We now confront the problems presented by the relations between these two kinds of antigen their similarities, their differences, and the significance of these relations It is in an attempt to clarify these questions that we summarize our work on autosensitization and the immunology of homografts, especially the phases of either problem that seem to be of interest in understanding the other

### Some Characteristics of Autoantigens and Autoantibodies

Our studies principally concerned testes and spermatozoa It is now well known that one can induce testicular lesions in guinea pigs by injecting them with homogenates of testes, from the same or another guinea pig, mixed with adjuvants When we first described this phenomenon in 1951,[3] we did not think that we were justified in ascribing it to the action of autoantibodies, principally for the reason that similar lesions can be induced in nonspecific ways, although usually less frequently and less significantly[4, 5] Subsequently we sought other tests of autosensitization to testis and spermatozoa, that is

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immobilization of autospermatozoa and isospermatozoa by autologous or homologous antiserum, general anaphylaxis, microprecipitation (ring) tests, and passive cutaneous anaphylaxis (PCA)

## Materials and Methods

### *Animals*

For these experiments we used more than 1000 guinea pigs (injected for sensitization, used for preparing extracts of testis, kidney, and spermatozoa, or for PCA tests) We also used rabbits, rats, and mice either for immunization or for challenging procedures

### *Immunization*

This was effected by means of organ homogenate preparations Insofar as possible, organs were deprived of blood and of connective capsules and blood vessels They were usually ground in a mortar with an equal amount of saline and then filtered through gauze, after which they were incorporated in a water-in-oil emulsion with regular Freund's adjuvants (1 part for 2 parts of homogenate) or added to aluminum hydroxide (1 part for 8 parts of homogenate) Occasionally we used a suspension of spermatozoa (about $5 \times 10^8$ per cc ) instead of organ homogenate Animals were usually injected by the intradermal route, but at other times subcutaneously or intraperitoneally, with 0 5 cc of the preparation The injections were repeated weekly as many as 4 to 10 times In addition to testes and spermatozoa, control organs used were kidney, liver, heart, brain, lens, and cornea A slightly greater number of animals were used as controls

### *Pathological Examinations*

These tests were done by the usual techniques, but because of the difficulties inherent in the specificity of the observed lesions such tests were not used systematically

### *Challenging Tests*

This group included spermatozoa-immobilization tests, general anaphylaxis, PCA, and microprecipitation (ring) tests

*Spermatozoa-immobilization test* With this test also, we often observed a nonspecific phenomenon, that is, the immobilization of spermatozoa by fresh sera from guinea pigs either uninjected or injected with unrelated organs However, this difficulty may be avoided in either of two ways The first of these makes use of the fact that after several weeks of storage at 4° C the nonspecific cytotoxicity disappears, while the presumed immune cytotoxicity persists As for the second method, we observed that the nonspecific cytotoxicity, as well as the presumed immune one, disappeared after heating for one hour at 56° C However, on adding progressive doses of complement to the heated sera, we noted that the presumed immune toxicity returned when a smaller dose of complement was used than did the nonspecific ones The

suspension of spermatozoa under study was then added in small quantities to the sera, which were treated as first described above, and samples were taken every minute for microscopic examination The proportion of surviving spermatozoa and the survival time of the remainder were evaluated

*General anaphylaxis test* We have already described in detail the technique that we used,[6] which consisted, in brief, of injecting saline extracts of organs or spermatozoa to experimental or control animals intravenously

*Passive cutaneous anaphylaxis (PCA) test* We used this test, described by Ovary,[7] with slight modifications One carefully shaves the back of young white guinea pigs weighing between 250 and 300 gm On the next day from 4 to 6 intradermal injections were done on the backs of these animals on either side of the spine and about 15 mm from it These injections were done with $\frac{1}{10}$ cc of the various sera under study After 16 hours (6 hours in the first experiments) these animals were injected intravenously with a mixture made of Evans blue dye (0 6 cc of a 5 per cent solution) and organ extract (1 cc in saline) This organ extract was about 5 times more diluted than when used for general anaphylaxis Frequently extracts or, on occasion, suspensions of spermatozoa, were used, the concentration corresponded (very roughly) to $10^8$ spermatozoa per cubic centimeter The animals were then sacrificed 15 min after intravenous injection, their skin was removed, and the results were read from the undersurface of the skin Whenever an antigen-antibody reaction occurred on the site of an intradermal injection Evans blue dye was allowed to pass through the dermal tissue, where it formed a blue spot Such skins can be kept intact for a period of years

*Microprecipitation (ring) tests* Small tubes (30 mm in length and 2 5 to 3 mm in inside diameter) were used Sera were placed in the bottom of the tubes with micropipettes, and the organ extracts chosen as antigens were placed very gently on the superior part of the tubes Interfacial precipitation was checked hourly, the best results were seen after 2 or 3 hours Since it is important that the sera as well as the organ extracts be completely clear, they they were centrifuged for 1 hour at 10,000 g The use of polyvinylpyrrolidone at the final concentration of 2 5 per cent may sensitize the reaction, provided it is first added to each of the reagents and the reagents are centrifuged at the same time and speed before being allowed to react. We sometimes complete the reaction by testing the possibility of absorbing antibodies by the corresponding antigens The organ suspension used for that purpose is 10 times more concentrated than that used for the test, the latter was 50 times as diluted as the homogenate used for sensitization After 1 hour of incubation at 37° C the mixture of serum and antigen was centrifuged for 1 hour at 10,000 g, and the supernatant was used as absorbed serum These tests were not always used simultaneously but, except in one instance, PCA and ring tests were always used

## Results

By injecting guinea pigs with guinea pig testis homogenate or guinea pig spermatozoa mixed with Freund's adjuvants one can induce testicular lesions,

ANTIORGAN ISOSENSITIZATION

1st Step: Intradermal injections: serum 1/10 cc

Sera from guinea pigs injected with:

Homologous testis (IT)

Homologous kidney (IR)



2nd Step: Intravenous injections of:
(16 hours later) EVANS blue + antigen
Antigen = guinea pig spermatozoa extract

FIGURE 1 Passive cutaneous anaphylaxis (PCA) Sera (IT) from guinea pigs injected with guinea pig testis (isoantigens) contain antibodies that can react with isoantigens from the same organ (extract of guinea pig spermatozoa) Sera (IR) from guinea pigs injected with homologous kidney unable to react with extracts of guinea pig spermatozoa Note that testes are used for sensitization and extracts of spermatozoa for testing only for reasons of technical convenience

as shown previously by ourselves[3] and by Freund *et al* [8] Furthermore, one can show sensitization and antibodies against extracts of guinea pig testis or spermatozoa by specific immobilization of the spermatozoa, by general anaphylaxis, by PCA (FIGURE 1), and by the microprecipitation test Freund's adjuvants are not required for anaphylactic sensitization, as observed by ourselves[3] and by Freund *et al* [9] In our experiments the ring test showed that these antibodies are specifically absorbed by extracts of guinea pig testis or spermatozoa However, this indicates only the possibility of inducing antitestis and antispermatozoa isoantibodies by using isoantigens

One can go further and consider the case of true autoantibodies, that is to say, antibodies that react with substances originating in the same organism that elaborated them We were able to induce such antibodies by injecting guinea pigs with autotestis, isotestis, or heterotestis

*Autoantigens* from the animal's own testes are capable of eliciting the formation of autoantibodies, capable of reacting with the testes of the same guinea pig We were able to demonstrate this by testicular lesions, immobilization of spermatozoa, general anaphylaxis, the PCA test (FIGURE 2), and the microprecipitation test




FIGURE 2 Passive cutaneous anaphylaxis (PCA) A serum (A) from a guinea pig injected with cells from its own testes (autoantigen) contains an antibody that reacts with an autoantigen from the same organ (an extract of the spermatozoa of the same guinea pig) Sera from a noninjected guinea pig (serum N) or from a guinea pig injected with homologous kidney (serum IR) are not able to react with extracts of guinea pig spermatozoa The reaction obtained with serum IT has the same meaning as in FIGURE 1 The difference in size and intensity of the two reactions does not bear any general meaning, for it was not found in other similar instances

*Isoantigens* from the testes of other guinea pigs can elicit the formation of autoantibodies We demonstrated this by testicular lesions, the PCA test, and the microprecipitation test

*Heteroantigens* from testes from animals of other species (that is, from rabbits, rats, and mice, alternately injected into the same guinea pigs) are able to elicit the formation of autoantibodies We showed this by the PCA test (FIGURE 3) and the microprecipitation test, with specific absorption, in the case of the ring test, of those antibodies by extracts of guinea pig spermatozoa or testes However, we must mention that in this case, and for technical convenience, we did not usually use autotestis but isotestis as the challenging antigen

Correlatively, an animal injected with its own testis (that is, with autoantigens), will produce antibodies We have found that these antibodies react with autotestis, isotestis, and heterotestis, in other words, they act as follows

As *autoantibodies*, as shown in FIGURE 2

As *isoantibodies*, immobilizing spermatozoa from other guinea pigs and react-




FIGURE 3 Passive cutaneous anaphylaxis (PCA) Right sera from guinea pigs injected with rabbit, rat, and mouse testis (heteroantigens) contain antibodies that react with isoantigens from the same organ (extracts of guinea pig spermatozoa) They also reacted with autoantigens (extracts of the spermatozoa of the same guinea pigs that had elaborated the antibodies) Left sera from guinea pigs injected with rabbit, rat, and mouse (heterologous) kidney do not react with extracts of guinea pig spermatozoa

ing with extracts of other guinea pig spermatozoa and testes in the PCA (FIGURE 4) and ring tests, and

As *heteroantibodies*, since such sera react to the PCA test (FIGURE 5) when challenged by an extract of rabbit spermatozoa However, we were unable to obtain positive results with the spermatozoa of rats and mice, but it is possible that we had too few animals to make sufficiently concentrated extracts We also found that sera from isoimmunized guinea pigs reacted to the PCA test (FIGURE 5) against an extract of a rabbit's spermatozoa We have not yet used tests other than PCA in this case

It thus becomes obvious that the autoantigen-autoantibody system with which we are concerned here is strictly organ specific, and that this organ specificity is completely independent of individual specificity and even, to a certain extent, independent of species specificity Reciprocally, these experiments show that an organ-specific antigen, independently of its individual specificity, can elicit the formation of an autoantibody

Several points should be noted before we begin any consideration of homografts Among 16 control sera from guinea pigs injected with homologous kidney, 4 reacted with homologous kidney extracts in the ring test This ability to react was specifically absorbed by incubation with kidney extracts, and, of course, was not exhibited by any of the sera originating in testes-immunized guinea pigs Furthermore, among 10 guinea pigs injected with homol-

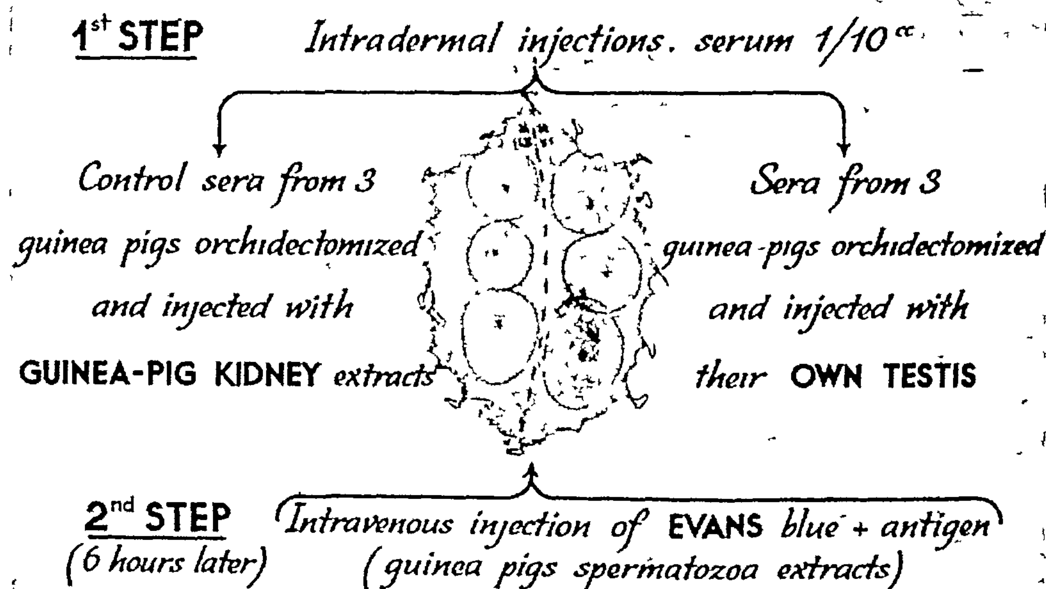



FIGURE 4 Passive cutaneous anaphylaxis (PCA) Right sera from guinea pigs injected with their own testes (autoantigens) contain antibodies that react with isoantigens from the same organ (extracts of guinea pig spermatozoa) Left sera from guinea pigs injected with guinea pig kidney do not react with extracts of guinea pig spermatozoa.

ogous kidney homogenate plus Freund's adjuvants, 4 exhibited anaphylactic shocks when challenged against homologous kidney extract in saline The controls were negative (testis extracts injected intravenously to 3 kidney-sensitized guinea pigs, and kidney extracts injected intravenously to 7 testis-sensitized guinea pigs) Among 18 guinea pigs each injected with one of its own kidneys plus Freund's adjuvants, then challenged with isokidney extract in saline, 2 exhibited a fatal anaphylactic shock and 2 others exhibited moderate anaphylactic shock, in 17 controls injected intravenously with the same extract no shock was observed The preceding results are the only positive ones that we have obtained thus far in the course of numerous experiments performed in order to confirm and extend the investigations of the Caveltis [10-12]

When using guinea pigs (or sera from guinea pigs) injected for sensitization with adjuvants plus organ homogenates such as heart, brain, lens, skin, and cornea the results were always negative when the sera were challenged against the corresponding organ extracts in saline or by PCA and ring tests Some positive results were obtained with skin and brain by general anaphylaxis [6]

## Some Aspects of the Immunology of Homografts

The experiments were conducted on the following bases Since it is known that spermatozoa have strong organ specificity, we considered it of interest to learn whether such cells also possess the individual specificity involved in homograft rejection We also recognized the importance of extending our

ORGAN SPECIFICITY OF ANTISPERMATOZOA AUTOANTIBODIES

(Auto & isoantigens ⟶ Heteroantibodies)

1st Step: Intradermal injections: serum 1/10 cc

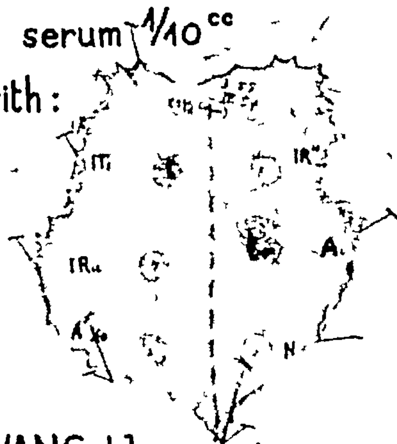
Sera from guinea pigs injected with:

Autologous testis (A)

Homologous testis (IT)

Homologous kidney (IR)

Nothing (N)




2nd Step: Intravenous injection of EVANS blue + antigen

(16 hours later) Antigen = Rabbit spermatozoa extract

FIGURE 5 Passive cutaneous anaphylaxis (PCA) Sera from either a guinea pig injected with its own testis (autoantigen A), middle right and lower left at a dilution of 1 20, or from a guinea pig injected with testes of another guinea pig (isoantigen IT) contain antibodies that react with rabbit spermatozoa extract (heteroantigen) Sera from either a noninjected guinea pig (N) or from a guinea pig injected with homologous kidney (IR) do not react with rabbit spermatozoa extract

knowledge of the kinds of tissues and cells that are able to induce immunity or tolerance to homografts For these reasons the prospective graft recipients (rabbits) were injected with spermatozoa or leukocytes from the prospective skin donor or from an unrelated animal They were skin-grafted after a short period of time, and the evolution of the grafts was observed

## First Series of Experiments

### *Materials and Methods*

Seventeen rabbits of a noninbred but close strain called *Fauves de Bourgogne* were used Fourteen animals served as skin recipients, of which 12 were preinjected with cells (leukocytes or spermatozoa) from 1 of 2 donors Next, the recipient animals were grafted with skin from two donors, one of which was a cell donor, the other being a new animal

The leukocytes (principally polymorphonuclears) from the 2 cell donors were obtained from peritoneal exudates that were formed after the intraperitoneal injection of sterile broth 2 and 4 hours previously The cellular suspensions were centrifuged (5 minutes at 1000 rpm), washed in 40 cc Hanks' solution, recentrifuged, resuspended in Hanks' solution and counted

The spermatozoa were obtained by the surgical removal and perfusion of epididymis and vas deferens after excision of the blood vessels They were washed in saline, perfused with Hanks' solution, and then treated in the same way as were the leukocytes

Twelve recipient animals were each immediately injected intradermally with ten million living cells, as follows three received leukocytes from one of the two future skin donors, three received spermatozoa from the same animal, three received leukocytes from another donor, and three received spermatozoa from the same animal that had donated the leukocytes to the preceding three animals Two prospective recipients, used as controls, received nothing

Six days later each of the 14 preceding rabbits received 2 skin grafts the first from the first cell donor, the second from a new and unrelated donor The technique used for these suprapannicular grafts on subpannicular beds has been described elsewhere *

Observations of the grafts were made by palpation and by the naked eye, and the general aspect of the graft, color, temperature, consistency, and state of fixation to the graft bed at the periphery was noted Such examination gives sufficient gross information concerning the state of the epithelium, the degree of vascularization and thrombosis, and the general vitality of the graft With the technique utilized here a first-set graft becomes dry and necrotic in about 12 days, a second-set graft in about 8 days Nevertheless, the exact end point is difficult to determine and, after thousands of grafts, we have found it best carefully to examine the grafts on the ninth day postgraft At that time a first-set graft is still alive, and a second-set graft is dead On that day the grafts are examined without knowing whence they come or what treatment the recipient received They are then rated according to their appearance as follows 4+ or Excellent, pink in totality, supple, and indistinguishable from an excellent autograft, 3+ or Good, but with some desquamation and/or a slight degree of cyanosis, 2+ or Medium, that is, with cyanosis, extensive desquamation, and/or brown appearance of part of the graft, an aspect denoting thrombosis in most of the vessels, 1+ or Poor, dry and brown for most of the graft, only a few points apparently remaining alive, and 0 or completely dead, necrotic, dry, and brown or black

*Results*

Examination of the grafts on day 9 gave the following results (average aspect) control grafts (4 grafts) all 3+, after specific leucocytes (3 grafts) 1, 3+, after nonspecific leukocytes (9 grafts) 2, 2+, after specific spermatozoa (2 grafts) 2, 5+, and after nonspecific spermatozoa (4 grafts) 2, 5+

This shows that specific leukocytes, but not specific spermatozoa, confer

* An unfortunate error occurred in our contribution to the *Second Tissue Homotransplantation Conference*[13] Concerning the technique of collecting the cells used for transferring passive (or "adoptive") hypersensitivity to the graft, on page 1064, line 19, instead of "The lymphocytes were collected by dissociation of *ganglia in* the spleen of the first guinea pig host" read "The lymphocytes were collected by dissociation of lymph nodes (*ganglions* in French) and spleens of the first guinea pig hosts" We must add that the lymph nodes that drain the site of the graft were always included, and that the word "lymphocytes" is used with the meaning of "lymph node cells"

immunity to the graft donor However, another interesting finding was that 2 female rabbits that had received spermatozoa exhibited 1+ grafts on the ninth day, regardless of the specificity or nonspecificity with respect to the donor of skin and spermatozoa This raised the question of the influence of the spermatozoa as immunizing agents in females against male grafts The following set of experiments was undertaken to settle this question

## SECOND SERIES OF EXPERIMENTS

### *General Procedure*

The general procedure for such experiments is divided into 2 stages (FIGURE 6) The first step consists of sensitization, 1 donor rabbit gives leukocytes to 2 recipients (1 male and 1 female) and spermatozoa to 2 recipients (also 1 male and 1 female) Two prospective skin recipients (1 male and 1 female) do not receive cells The second step consists of grafting All of the 6 preceding rabbits receive 3 grafts, 1 from the cell donor, 1 from another nonrelated male rabbit, and 1 from a female rabbit The grafts are examined on days 7, 9, and 11 and rated on day 9 Six such experiments were performed

### *Technique*

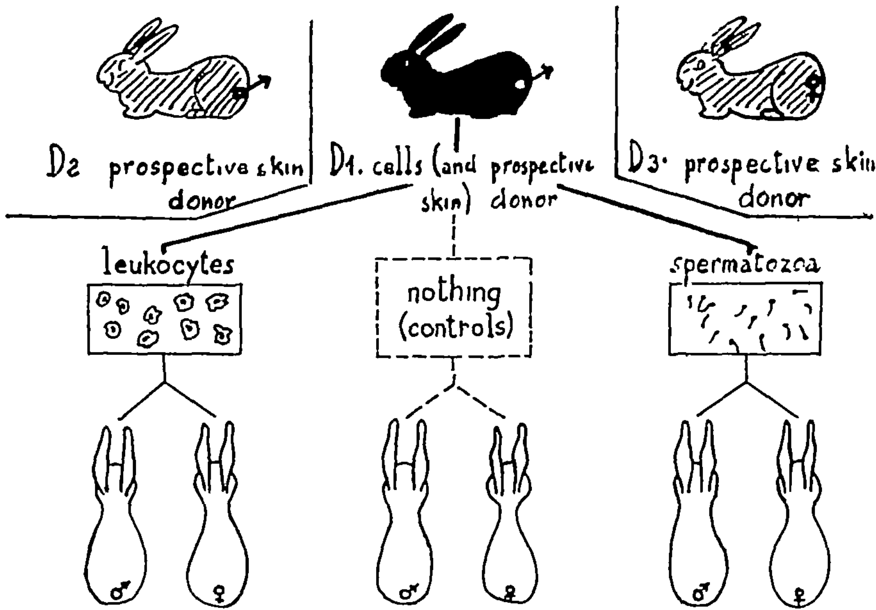
The procedure was essentially the same as in the preceding set of experiments The differences are as follows

Sensitization is done intradermally in 3 stages The first time half of the cells (living) are injected The second half is kept frozen (−20° C) and is injected in 2 equal doses, 1 on the fourth day and 1 on the sixth day Grafting is done on the eighth day after the first injection

The number of spermatozoa and leukocytes was the same in each set or in each half of a set (one half each of both males and females) This number varied from 6 million to 65 million except in one instance in which 250 million spermatozoa were injected

### *Results*

The general results are expressed in FIGURE 7 One must point out that in some groups the results are fairly homogeneous, while in other groups they are not This indicates the need for considerable caution about their interpretation. Nevertheless, there are 3 points that seem to be consistent First, specific leukocytes (that is, those from the same donor as the graft) immunized males and females against the graft, while nonspecific leukocytes did not, a fact already known[14] Second, neither specific (FIGURE 8) nor nonspecific spermatozoa seemed to immunize males against the graft Finally, an unexpected but parallel result is that female grafts on males had shorter survival times than did male grafts on females Of less consistency seem to be the 3 following points (1) spermatozoa appeared to sensitize females to male skin grafts in some instances, such as those shown in FIGURE 8, there being no difference in this respect between specific and nonspecific spermatozoa, (2) females seemed to be better recipients (2, 8+) than males (2, 1+), but (3) males seemed to be better donors (2, 6+) than females (1, 9+)



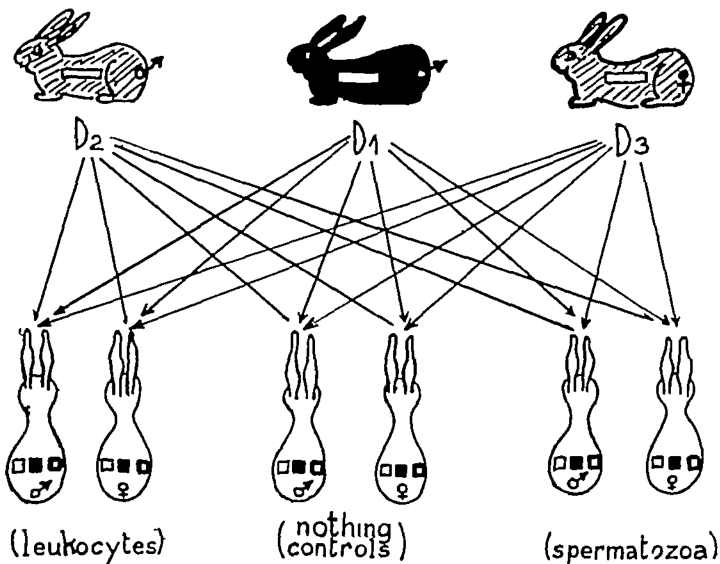
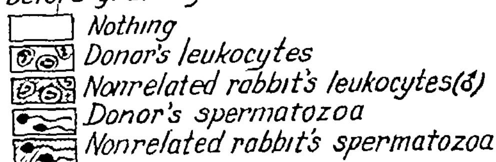




FIGURE 6 Diagram of experiments dealing with sensitization toward the graft to study individual and sex specificity and the presence or absence of individual specificity in spermatozoa



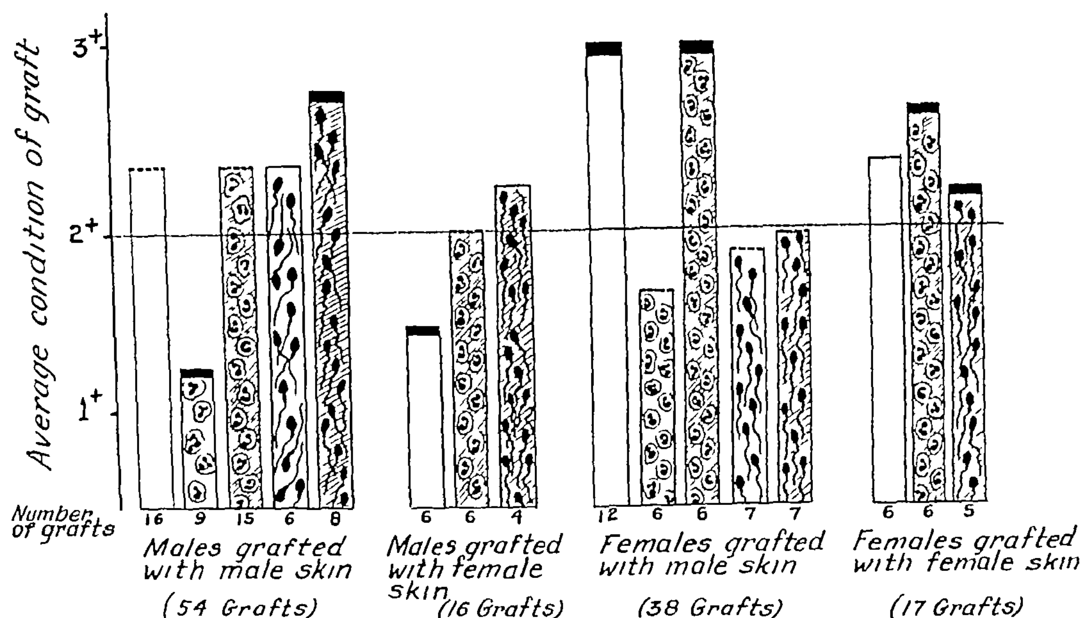




FIGURE 7 Graph showing the results of the preceding experiments The appearance of the grafts is evaluated from 0 to 4+ according to the procedure described in the text

## Discussion

In order to appreciate the significance of the results obtained, it is necessary first to appreciate the value of the techniques utilized

### *Criteria for Autosensitization*

*Pathological test* [3, 8] We have noted above that nonspecific lesions of the aspermatogenetic type could be induced by nonspecific aggression,[4] this may happen even when Freund's adjuvants are used alone [5] This does not mean that it is impossible to induce lesions of the same significance in testes as in the case of allergic encephalomyelitis A portion of the observed testicular lesions noted after injections of homologous testis is certainly due to such a phenomenon, especially when perivascular infiltrations are prominent Nevertheless, it appears that the pathological test should be interpreted very cautiously, and it is highly desirable to supplement such observations with other tests of autosensitization to testis and spermatozoa

*Immobilization of spermatozoa* In this case also one frequently may observe the immobilization of spermatozoa by fresh normal homologous sera It is thus impossible to rely upon the immobilization test unless one uses one of the two techniques described above in order to eliminate nonspecific spermotoxicity

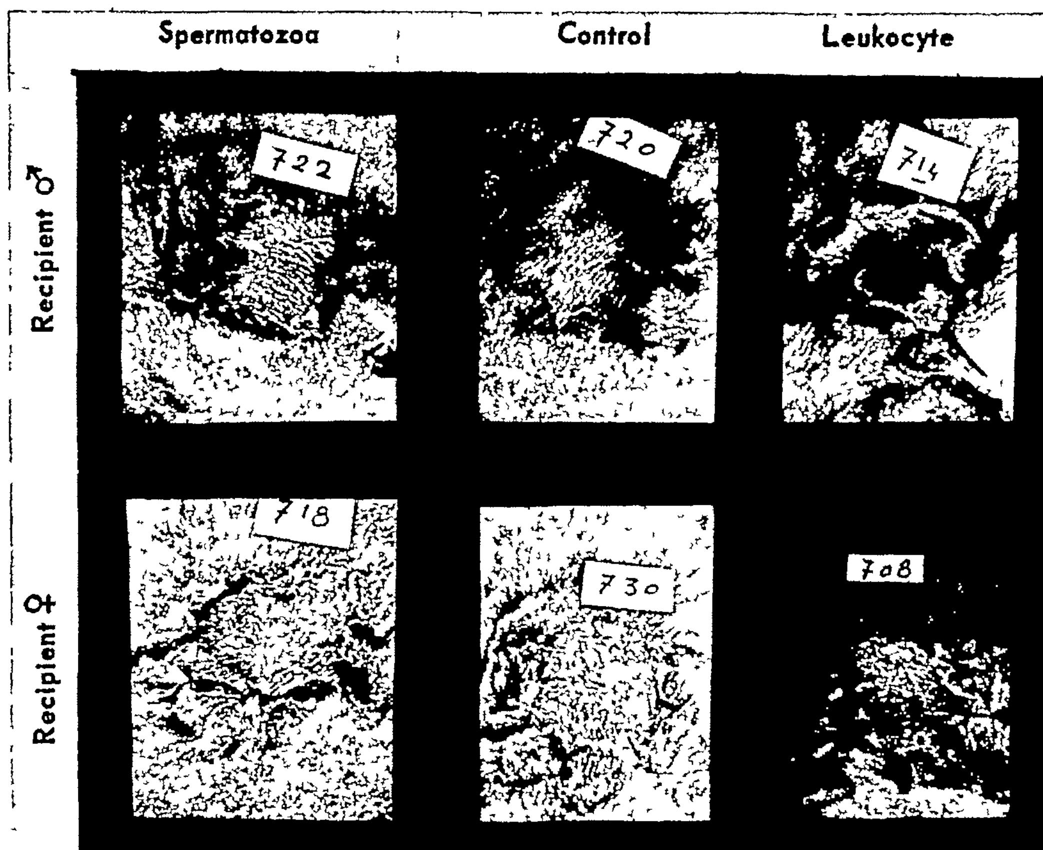



FIGURE 8 Appearance of skin grafts 9 days after grafting in one of the experiments The 3 upper grafts were made on male hosts, the three lower ones on female hosts Skin grafts were taken from a unique male donor whose spermatozoa previously had been injected into the 2 prospective hosts (left pair) and whose leukocytes had been injected into 2 other prospective hosts (right pair) Two other hosts (middle pair) received no previous injection The grafts in hosts previously injected with specific leukocytes are dead (upper right) or cyanotic and dry (lower right) All of the grafts in hosts previously injected with specific spermatozoa (left pair) are good, even the graft on the female host in this particular case

*General anaphylaxis* [6] This is a good test for autosensitization, provided the animals are healthy and sufficiently sensitized, and provided the extract injected intravenously is not too concentrated The general anaphylaxis test is particularly reliable in the case of autosensitization to testis or spermatozoa

*Passive cutaneous anaphylaxis* This technique appears to be the most satisfactory that we have used thus far because of its high sensitivity and specificity and because it permits one to make semiquantitative tests and several controls on the *same* reactive animal The tests can be kept in a book and compared to other tests performed months or years later

*Ring test* This is a good technique, provided the reagents (sera and organ or cell extracts) are clarified by high-speed centrifugation Nevertheless, the ring test seems to be of lower sensitivity than the PCA test, which does not require precipitations On the other hand, the ring-test technique allows experiments on specific absorption by organ extracts, which would be difficult

in PCA tests because centrifugation does not remove nonprecipitated antigen-antibody complexes and because organ extracts are usually proinflammatory, which might produce nonspecific reactions Finally, it is advisable systematically to associate the PCA and ring tests, either alone or together with other tests, principally in order to confirm the latter

### *Techniques Utilized for Homografts*

*The technique of grafting* described here has proved satisfactory in rabbits and guinea pigs, as indicated by numerous autografts and by the constant vascularization of first-set homografts [13]

*The technique of evaluation of the grafts*, however, must be considered somewhat more carefully The end-point technique does not seem satisfactory because of the difficulty of making a clear-cut evaluation of the survival time Even when using more precise methods, either histological or stereomicroscopic, this end point is difficult to establish precisely because thrombosis can occur in some points and not in others, and several days can elapse between the thrombosis first observed and general thrombosis of the graft A careful gross examination seems to provide at least as valuable information concerning vascularization, thrombosis, and general vitality of the graft as a whole While useful, the end-point technique is exceeded in accuracy by examination of the graft on a day chosen when a first-set homograft is almost always alive and when a second-set one is almost always dead In our experience this day is the ninth Using this technique, one may rate grafts from 0 to 4+ according to their appearance The observer must know nothing of the nature of either the donor or the recipient This procedure seemed to us to be more accurate than the end-point technique, since it does not prevent one from following the fate of the graft and estimating the apparent survival time In any case, this difficulty is not the principal one, which is inherent in the variability of the grafts

*Variability in aspects and survival times of grafts* This variability sometimes impairs the homogeneity of the results, but it seems unavoidable in strains that are not highly inbred It probably arises both from the variable vitality of grafts originating in different animals and from the variable susceptibility of different recipients toward the same donor It is not impossible that phenomena such as the hair cycle described in rats by Ballantyne and Converse[15] influence this variability in some way On the other hand, the question of sex seems to be significant for both donors and recipients, as described above Finally, it appears necessary always to use cross controls for the donors as well as for the recipients and to observe the utmost caution in interpreting the results.

### *Significance of the Results*

Several problems are involved in these experiments Nevertheless, we shall concentrate here on the results that are related to the problem that they were designed to clarify namely, the relationship between autoantigens and homograft antigens Three points deserve consideration in this respect

The first point is that autoantigens are organ specific and that the organ

specificity of an antigen and its ability to be autoantigenic seem to be very strongly linked This relationship seems to have been established in the case of spermatozoa Is there such a linkage in other known autoantigen-autoantibody systems? This appears to be the case although, to our knowledge, no systematic experiments in this direction have been attempted In the case of the white matter of the central nervous system it is known that allergic encephalomyelitis, which is thought to be due to a challenge of the autoantibody type, regardless of whether those antibodies are serum antibodies or the so-called intracellular antibodies, can be induced by injections of autoantigens,[16] of isoantigens (as in numerous experiments), or of heteroantigens [17,18] In the case of lens cells it has been demonstrated that the organ specificity of their antigens, which are able to induce autosensitization, is broadly independent of species specificity[19] and overlaps from dogs to cattle, for example It also has been shown that antibodies elicited by isolenses can react against extracts of lenses from other species [20] in other words, they are able to act as hetero-antibodies One of the implications of these facts is as follows [21] The ability of a given antibody formed against a heteroantigen or an isoantigen to react against a substance originating in the organism producing that antibody (that is, if this antibody is really organ specific), indicates that this substance possesses the same immunological determinant as the heteroantigen or isoantigen in question From this it follows that the original substance may be or can become autoantigenic and induce the formation of an autoantibody that, being organ specific, will be able to act as an autoantibody, as an isoantibody, or as a heteroantibody

The second point concerns the individual specificity of homograft antigens

The third is that spermatozoa possess organ specificity and do not seem to possess individual specificity Let us consider the implications of these facts in view of the nature of the two types of antigens and their interrelationship

*Autoantigens* Since they are organ specific (in a broad sense*), their sites of action are limited to an organ, a tissue, a functional system, or a definite category of cells and do not extend to other tissues or cells of the body In turn, these autoantigens are shared by all of the individuals of the same species, and even of some other species (in male animals, for example, this would be true of spermatozoa) Therefore they seem probably linked to substances related to a special functional activity that does not consist of the general process of synthesis of specific proteins, but of intermediate metabolisms The same organ (or, rather, functional system) of any individual in one strain, one species, or even in several species) is able to perform the same function apparently without remarkable differences between individuals Furthermore, it does not seem important, as far as autoantigenicity is concerned, whether the tissues of cells are alive, dead, or extracted What is the chemical nature of these substances? One theoretical point must be emphasized the desoxyribonucleic acid–ribonucleic acid (DNA-RNA) system, which depends directly upon the chromosomal constitution of the cell and is thus strictly and completely determined by the genetic constitution of its parental ovum and spermatozoon, will not normally build proteins foreign to the organism It seems difficult

* It would be even better to speak of function specificity

to admit that this synthesizing mechanism can build proteins that in turn, can induce the same mechanism to build antibodies against them Consequently it is unlikely that normal "standard" proteins can be autoantigenic regardless of any question of specific "naturally acquired" tolerance, and that autoantigens can either derive from "nonstandard" proteins (for example, proteins modified by denaturation or by the presence of a prosthetic group) from nonprotein material This appears to be the case for autoantigens to spermatozoa We have localized the organ specificity of spermatozoa in a fraction that is soluble in trichloracetic acid, heat stable (1 hour at 100° C) and nondialyzable This agrees with the finding of Freund *et al* of aspermatogenetic activity in a somewhat comparable fraction of homologous testis extracts [9]

*Homograft antigens* Contrary to the situation with autoantigens, homograft antigens are not shared by different individuals of one species On the other hand, in a given individual they are shared by most of the cells that have been tested thus far, at least this is the case with cells that can actively synthesize proteins and can reproduce themselves These substances are not related to any particular functional aspect of any organ, but they seem to derive from the intimate structure of the protein-synthesizing mechanisms Furthermore, homograft antigens have not been found outside living cells which alone induce homograft tolerance and/or homograft immunity or, at least, outside of substances related to the protein-synthesizing mechanisms that can induce immunity Therefore, without taking a position as advanced as that of Medawar[22] one can infer that homograft antigens are related in some way to the protein-synthetizing mechanisms, and that nucleoproteins thus represent merely the most evident part of them

*The relationship between autoantigens and homograft antigens* The individual specificity that results in homograft antigenicity seems to be precisely the factor that prevents an antigen from being autoantigenic [21] On the other hand, organ specificity, which seems to be linked to autoantigenicity, does not induce immunity against a given individual, and therefore it cannot induce homograft immunity The relationship between organ specificity and individual specificity can best be studied through spermatozoa, for these possess the ideal property of being organ specific without having (or at least without seeming to have) individual specificity Why should this be the case? Why are spermatozoa organ specific and why should they not be individual specific? Let us offer a tentative explanation the organ specificity of spermatozoa may be due to a substance linked to the special function of motility possessed by these cells In support of this hypothesis, we have obtained some positive results with isolated tails of spermatozoa As for individual specificity, it might well be that individual-specific antigens, whatever they are, appear only when the individual itself appears, that is, after fertilization of the ovum by the spermatozoon and the reconstitution of the normal diploidy of the somatic cells at the time when the specific synthesizing mechanisms suddenly begin to function Nevertheless, one must not be overly systematic, and it would be well to add some considerations concerning the antigens that are involved in the rejection of homografts The individual-specific antigens that elicit homograft immunity may not be the only ones involved, especially in some experimental

cases For instance, we have previously reported[13] the rejection of autografts in two instances first, in rabbits injected with rabbit skin plus adjuvants and exhibiting a dermatosis attributed to organ (skin) sensitization and, second, in guinea pigs injected with rabbit antisera against guinea pig skin These rejections indicate that organ-specific sensitization and species-specific antibodies can influence the rejection of a graft in some way However, such situations are highly artificial, and the antibodies (either serum antibodies or so-called intracellular antibodies, whatever they may be) that seem to cause the rejection of the graft are not at all specifically directed against the graft but rather against the entire skin of the animal However, such antibodies are more harmful at the graft site because the grafting procedure itself handicaps survival of the graft[23] Another case in which an individual-specific antigen would not be involved is that of sex specificity and Y chromosome[24] However, our consideration of the questions arising from differences in the behavior of males and females as donors or recipients of skin and the possibility of sensitization of females to male skin by spermatozoa must be brief, for two reasons first, the results were not sufficiently consistent and, second, it would be wise to consider the possibility of hormonal mechanisms before speaking of "sex-specificity" in that connection In any case, it appears that the term individual-specificity has, as far as homograft antigens are concerned, a relative meaning in relation to the individual differences between the donor and the recipient, more precisely, the antigens present in the donor and absent from the recipient

## Conclusion

The results of these experiments lead us to the following two conclusions

(1) Autoantigens are in a broad sense organ specific, they are related to a functional activity, they are shared by every individual of a given species and, eventually, of other species, finally, they are not likely to be holoproteins

(2) Autoantigens and homograft antigens are mutually incompatible, that is to say, a homograft antigen cannot be autoantigenic, conversely, an autoantigen cannot be "homograft-antigenic"

## Summary

(1) Experimentally induced immobilisins, precipitins, and anaphylactic (active general anaphylaxis and passive local anaphylaxis) autoantibodies have been induced and demonstrated in guinea pigs by means of autologous or homologous extracts or homogenates of testes or spermatozoa

(2) Organ-specific antibodies against autologous or homologous kidney, homologous brain, and skin have been induced and demonstrated in several instances

(3) Autoantigens are organ specific that is, they have no individual specificity and, to a certain extent, they are independent of species specificity, as demonstrated by the fact that they can induce antibodies that act as autoantibodies, isoantibodies, or heteroantibodies Conversely, antibodies that act as autoantibodies can be induced by autoantigens, isoantigens, or heteroantigens

(4) Spermatozoa from a donor were unable to induce an accelerated rejection of a subsequent skin homograft from the same donor, contrary to our experience with leukocytes

(5) The results are discussed in view of the nature of autoantigens and homograft antigens and their relationship

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*References*

1 MEDAWAR, P B 1946 The fate of skin homografts transplanted to the brain, to subcutaneous tissue and to the anterior chamber of the eye Brit J Exptl Pathol. **29**. 58–69
2 ARON, M 1958 Greffes homoplastiques intratesticulaires chez le cobaye Coll Intern Biol Homogreffes Paris, France In press
3 VOISIN, G A, A DELAUNAY & M BARBER 1951 Sur des lésions testiculaires provoquées chez le cobaye par iso- et auto-sensibilisation Ann inst Pasteur **81**. 48–63
4 DELAUNAY, A & G A VOISIN 1952 Sur des lésions testiculaires provoquées chez le cobaye et chez le rat por l'endotoxine typhique Compt rend **234** 158–160
5 VOISIN, G A & A DELAUNAY 1955 Sur les lésions testiculaires observées chez des animaux soumis à des injections de substances adjuvantes seules ou mélangées avec des extraits de tissus homologues Ann inst Pasteur **89** 306–317
6 VOISIN, G & A DELAUNAY 1955 Mise en évidence d'un état d'iso- ou d'auto-sensibilisation au moyen du choc anaphylactique Ann inst Pasteur **89**. 556–581
7 OVARY, Z & M BRIOT 1953 Nouvelle méthode de dosage de l'anticorps anaphylactique et son rapport avec l'azote de l'anticorps Ann inst Pasteur **84**. 620–671
8 FREUND, J, M M LIPTON, & G E THOMPSON 1953 Aspermatogenesis in the guinea pig induced by testicular tissue and adjuvants J Exptl Med **97** 711–725
9 FREUND, J, G E THOMPSON & M M LIPTON 1955 Aspermatogenesis, anaphylaxis and cutaneous sensitization induced in the guinea pig by homologous testicular extracts J Exptl Med **101** 591–604
10 CAVELTI, P A & E S CAVELTI 1945 Studies on the pathogenesis of glomerulonephritis I Production of autoantibodies to kidney in experimental animals A M.A Arch Pathol **39** 148–152
11 CAVELTI, P A & E S CAVELTI 1945 Studies on the pathogenesis of glomerulonephritis II Production of glomerulonephritis in rats by means of auto-antibodies to kidney A M A Arch Pathol **40**· 158–162
12 VOISIN, G A 1955 Les auto-anticorps cytotoxiques Étude critique, clinique et expérimentale 30ᵉ Congr Français Méd Alger **2** 225–264 Masson Paris, France
13 VOISIN, G A & P MAURER 1957 Studies on the role of antibodies in the failure of homografts Ann N Y Acad Sci **64**(5)· 1053–1070
14 MEDAWAR, P B 1946 Immunity to homologous grafted skin II The relationship between the antigens of blood and skin Brit J Exptl Pathol **27** 15
15 BALLANTYNE, D L & J M CONVERSE 1957 The relation of hair cycles to the survival time of suprapannicular and subpannicular skin homografts in rats Ann N Y Acad Sci **64**(5) 958–966
16 KABAT, E A, A WOLF & A E BEZER 1949 Studies on acute disseminated encephalomyelitis produced experimentally in rhesus monkeys IV Disseminated encephalomyelitis produced in monkeys with their own brain tissue J Exptl Med **89**· 395–398
17 RIVERS, T M, D H SPRUNT & G P BERRY 1933 Observations on attempts to produce acute disseminated encephalomyelitis in monkeys J Exptl Med **58**. 39–54
18 RIVERS, T M & F F SCHWENTKER 1935 Encephalomyelitis accompanied by myelin destruction experimentally produced in monkeys J Exptl Med **61**: 689–702
19 MARKIN, L & P KEYES 1939 Species specificity of proteins of the optic lens J Infectious Diseases **65**. 156–159

20 HALBERT, S P, D LOCATCHER-KHORAZO, L SWICK, R WITMER, B SEEGAL & P FITZGERALD 1957 Homologous immunological studies of ocular lens I *In vitro* observations J Exptl Med **105**· 439–452
21 VOISIN, G A 1956 Le concept d'auto-anticorps (à la lumière des travaux expérimentaux récents) Rev hématol **11** 49–57
22 MEDAWAR, P B 1958 The antigenic stimulus in transplantation immunity Coll Intern Biol Homogreffes Paris In press
23 WAKSMAN, B H 1957 Second Tissue Homotransplantation Conference Ann N Y Acad Sci **64**(5) 1070–1073
24 EICHWALD, E J, C R SILMSER & N WHEELER 1957 The genetics of skin grafting Ann N Y Acad Sci **64**(5). 737–740

# EFFECTS OF PROTEIN DEFICIENCY AND MASSIVE INTERNAL IRRADIATION OF THE RETICULOENDOTHELIAL SYSTEM ON ANTIBODY REACTIONS IN KIDNEY HOMOTRANSPLANTATION*

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The production of hemoglobin occupies a high priority in the body economy of protein metabolism, this pigment is manufactured even at the expense of plasma protein under most circumstances Inability to form hemoglobin due to lack of sufficient protein intake is encountered clinically in the so-called "pernicious anemia of pregnancy," a condition relatively rare in all parts of the world except the Orient However, it has been possible to demonstrate the limitation of hemoglobin formation in dogs by restriction of dietary protein upon which is superimposed the "forced production" of this pigment [1] This latter has been accomplished by maintaining these experimental animals in a chronic state of anemia by means of repeated bleeding, thus stimulating the bone marrow to a proliferative state and, at the same time, administering by vein an amount of iron in excess of that removed by the equivalent amount of hemoglobin Under such conditions there were also found, along with the depletion of body reserve protein, reflected lowered plasma protein levels, with consequent marked alteration in the electrophoretic configuration of the latter [2] Modification of the anaphylactic response following sensitization of such dogs to horse serum was shown,[2] even though it is to be assumed that antibody formation is also likely to possess a high degree of priority in the body economy This suggested the possible application of the effect of marked protein depletion upon the production of antibodies and the relationship of such a state to the problem of homotransplantation The simplest procedure that could be studied seemed to be the application of skin homografts, and this has been investigated briefly [3] Survivals of skin homografts were of even shorter duration in the protein-depleted animals than in the normal control animals However, we do not think this should necessarily be attributed to the factors involved in antibody production As is well known, the normal early maintenance of skin homografts is accomplished by nutrition of the graft through imbibition until the time when adequate vascularization of the graft may be developed through collateral circulation In the dogs studied under these conditions of protein depletion, there was deposited a deep layer of subcutaneous fat of low melting point in spite of the high degree of emaciation present in most of these animals It was thus felt that, in their early stages, the homografts were deprived of the required nutriment necessary for their temporary establishment For this reason we feel that the skin homograft technique is not likely to provide us with the desired information concerning the donor-host antibody relationships under the experimental conditions used

Protein-depleted dogs maintained under similar conditions were subjected

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to kidney homotransplants In some cases only the recipient was a protein-depleted animal, in others both donor and recipient were protein depleted According to the views of Dempster[4] and of Simonsen,[5] not only does the host react against the organ, but the latter reacts against the host It was to cover such an exigency that we included groups of experimental dogs in which the donor was subjected to protein depletion or irradiation

It is felt variously by investigators that (1) the reticuloendothelial cells, (2) *the plasma cells, or (3) the lymphocytes are mainly responsible for the formation of antibodies* An interesting brief discussion of the roles that various types of cells (for example, macrophages, plasma cells, and lymphocytes) play in the formation of antibody, and the evidence for each, has recently been offered by Wissler *et al* [6] On the other hand, these investigators, as well as some others, believe that the primitive reticular cell, which is generally not considered phagocytic, is probably, of all cells, the most likely to be implicated in antibody formation

It has been shown by Taliaferro and Taliaferro that whole-body irradiation with X rays resulted in the depression of antibody formation [7] When radioactive gold colloids are administered by vein they are selectively taken up by the reticuloendothelial system (RES) and thus permit very intensive irradiation of that system If antibodies are formed largely by RE cells, then it might be expected that such irradiation would produce alterations within the scope of experimental observation [8] Furthermore, if plasma cells are derived from the reticuloendothelial tissue, massive irradiation of the latter might be expected to result in the reduction of the number of plasma cells formed and there would be a limitation of antibody production Finally, the induction of lymphopenia following irradiation in general and massive irradiation of the RES, including the hematopoietic system, has been very commonly observed by us [9] Therefore, it might reasonably be predicted that such massive internal irradiation of the RES would affect the production of antibodies

## *Methods*

Dogs were maintained on a diet, described elsewhere,[1] that consisted largely of bananas, the protein contribution of which represented 7 5 per cent of the total caloric intake The animals were initially rendered anemic by the removal of one quarter of the estimated total blood volume (72 ml /kg ) on 3 successive days and an additional one quarter on the fifth day Subsequently, such bleeding was effected as to maintain the hematocrit level at from one third to one half of normal Following each phlebotomy, iron* was administered intravenously in such an amount that 1 mg of iron was administered for each milliliter of erythrocytes removed In the normal animal this could constitute a rough balance and, in the anemic animal, this actually represents a surplus of iron administered, because of hypochromia

Radioactive colloidal gold† was administered by vein or artery in undiluted form

* Saccharated iron (Proferrin) was provided by Merck Sharp & Dohme, Philadelphia, Pa

† Radioactive gold colloid was obtained from Abbott Laboratories, Oak Ridge, Tenn

Homotransplants of kidneys were all carried out to the neck of the recipient *
Following induction of anesthesia, using sodium pentobarbital, the donor animal was heparinized by the intravenous administration of 10 mg of heparin In those instances in which the donor had received massive amounts of internal irradiation and where it was planned subsequently to use the second kidney from the same animal, postnephrectomy bleeding due to the heparin levels in the blood was counteracted by the administration of 10 mg of protamine sulfate

In those animals in which radioactive material was to be injected in the carotid vessels following transplantation of the kidney, a near closure of the overlying skin was made prior to the injection in order to minimize exposure subsequent to the injection This necessitated leaving only a small part of the arterial supply to the transplanted kidney approachable, a step requiring only a few sutures after administration of the gold

In recipient animals that had recently received large doses of internal radiation the hepatic and splenic areas were shielded with leaded-rubber protective pads to minimize exposure to operators

Daily observation as to urine formation and the presence or absence of hemoglobinuria was made When anuria was encountered, the kidney was removed and histological sections were taken

*Experimental Observations*

Functioning time in days represented the period from operation until the transplanted kidney became anuric This condition was ordinarily heralded by hemoglobinuria perhaps one or two days preceding renal shut-down

Very often, the polyethylene tubing used as a ureteral catheter was "scratched out" or otherwise dislocated by the animal, but reinsertion was usually possible Its chief purpose was for observation of secretory flow rate When the catheter could not be replaced, the stab wound through which it led usually retained patency, so that function was maintained

TABLE 1 shows the times of functional survival of a group of control animals The survivals, ranging from 3 to 6 days, are in keeping with the results of other investigators TABLE 2 shows survivals of the transplants in the protein-depleted recipients and animals in which both donor and recipient were depleted The first 4 recipients, dogs No 56-162 to No 56-165, were litter mates The donors, as well as the recipients (dogs No 56-171 to No 56-175) were litter mates Donor No 56-198 and recipients No 56-197 to No 56-201 were also litter mates In the protein-depleted group the survivals are not sufficiently greater than in the control group to warrant speculation

Use of intravenous radioactive colloids enables the investigator selectively to irradiate the reticuloendothelial system at limitlessly high ranges In some instances the prospective donor animals were injected with amounts of radiogold that were sufficient to result in death by acute radiation within 10 days,[8] the kidneys were usually removed for transplantation in one week or less In

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TABLE 1
HOMOTRANSPLANTATION OF NORMAL DONOR KIDNEYS INTO NORMAL RECIPIENTS

| Donor | Recipient | Au$^{198}$ Functioning time (days) | Remarks |
|---|---|---|---|
| 57-25 | 57-23 (F) | 5 | |
| 57-58 | 57-59 (F) | 4 | |
| 57-57 | 57-56 | 3 | |
| 57-90 (F) | 57-91 | 5 | |
| 57-95 (M) | 57-94 | 6 | Heparinized with 30 mg of heparin |
| 58-5 (F) | 58-6 (F) | 5 | Transplanted kidney very nodular, extensively covered with perirenal fat, white, glistening scarlike tissue on lateral surface Heparinized with 30 mg of heparin |

TABLE 2
PROTEIN-DEPLETED ANIMALS

| Donor | Recipient | Functioning time (days) | Remarks |
|---|---|---|---|
| VAH-157 | 56-164* (M) | 3 | Donor kidney was not heparinized |
| VAH-257 | 56-163* (F) | 6 | On second day wound was found open, it was then closed with 2 uninterrupted sutures |
| VAH-357 | 56-162* (F) | 15 | On sixth day wound was found open, it was then closed with 2 uninterrupted sutures On the twelfth day polyethylene tubing was reinserted On the fourteenth day 2 more sutures were used in closing wound |
| VAH-457 | 56-165* (M) | 11 | Donor transfused during nephrectomy with whole blood On postoperative day 6, 2 uninterrupted sutures were employed for wound closure On postoperative day 10, the animal attempted to scratch out the transplant, transplant showed evidence of hemoglobinuria On the eleventh postoperative day the transplant was damaged by scratching |
| 56-171* | 56-174* (F) | 6 | |
| 56-175* | 56-172* (F) | 7 | |
| 56-198* | 56-197* (F) | 5 | |
| 57-09 | 56-200* (F) | 7 | |
| 57-44 | 56-201* | 4 | |

* Protein-depleted

another group of animals only the recipient received injection into the afferent artery immediately following transplantation of the kidney

Equivalent roentgen dosages were estimated from the formula of Marinelli *et al*,[9] assuming that the liver represented 2 4 per cent of the body weight and that 90 per cent of the colloid was taken up by this organ

In the course of other studies on the production of cirrhosis and ascites in dogs[8, 10-12] following massive amounts of internal irradiation of the RES we have accumulated a considerable amount of hematological data We should like to stress that these radiation levels are 10 or more times those used in

TABLE 3

DONOR PREVIOUSLY INJECTED WITH MASSIVE AMOUNTS OF $Au^{198}$ VIA JUGULAR VEIN

| Donor | $Au^{198}$ Administration (mc ) | Recipient | Functioning Time (days) | Remarks |
|---|---|---|---|---|
| 57-37 | 92 | 57-39 | 3 | Right nephrectomy was performed on donor 6 days after administration of Au |
| 57-64 | 142 | 57-48 | 9 | Seven days after injection of Au, left nephrectomy performed |
| 57-64 | | 57-53 | 3 | Right nephrectomy performed 9 days after Au injection into donor |
| 57-73 | 181 | 57-77 | 7 | Left nephrectomy 6 days after Au injection into donor |
| 57-73 | | 57-78 | 5 | Right nephrectomy 6 days after Au injection into donor |
| 57-51 | 159 | 57-46 | 19 | Left nephrectomy 9 days after Au injection into donor Recipient showed signs of anorexia on thirteenth day (500 cc of 5% glucose sol ) and 500 mg of terramycin was administered on sixteenth day Same on eighteenth day Died on nineteenth day |
| 57-51 | | 57-62 | 4 | Right nephrectomy on ninth day after Au injection into donor |
| 57-42 | 150 | 56-177* | 8 | |
| 57-42 | | 56-178* | 4 | Abnormal anatomic finding 2 renal arteries to right kidney Anastomosis took 2¾ hours |

* Protein-depleted

therapy by the intravenous route in human disease These larger single experimental doses (150 mc ) usually result in death in 1 to 2 weeks in young dogs, but are better tolerated in adult dogs The degree of lymphopenia is often quite marked, but is transient The temporary anemia has never been explained satisfactorily since, in view of the lifespan of the red blood cells, interference with formation of the erythrocyte may probably be excluded, since its results would not become apparent at such an early stage

Of the series in which the donor was subjected to massive irradiation previous to nephrectomy (TABLE 3) only one recipient tolerated the transplant for any extended period This dog, No 57-46, died on the nineteenth postoperative day before the kidney became anuric

In TABLE 4 it can be seen that there were no notable increases in functional survival where the kidney was injected by the arterial route immediately after transplantation This route was used in order to allow maximal uptake of colloid by the RE cells of the kidney However, less than 0 1 per cent of the injected material was found resident in that organ on several occasions, the effective irradiation being confined to the RES of the recipient

Since histological studies are incomplete at this time, incidence of retrograde pyelonephritis and other sequellae such as degree of edema and necrosis of the transplant will be described elsewhere

The most striking difference noted in animals that had received arterial injection of radiocolloids to the transplant, as contrasted with the normal and

TABLE 4
NORMAL MONGREL DOGS WITH KIDNEY HOMOTRANSPLANT IRRADIATED WITH $Au^{198}$ VIA CAROTID ARTERY

| Donor | Recipient | $Au^{198}$ Amt Adm (mc.) | Functioning time (days) | Remarks |
|---|---|---|---|---|
| 57-38 | 57-17 (M) | 50 | 5 | Polyethylene tubing scratched out on first and second days after transplant, replaced on both occasions |
| 57-28 | 57-36 (F) | 50 | 7 | |
| 57-15 | 57-6 (F) | 50 | 8 | Urine remained clear after tubing replacement On fourth day polyethylene tubing scratched and replaced |
| 56-185 | 57-20 (F) | 80 | 3 | On third day animal scratched out entire transplant |
| 57-49 | 57-50 (F) | 54 | 7 | |
| 57-47 | 57-65 (F) | 48 | 6 | |
| 57-75 | 57-72 (F) | 52 | 7 | |
| 57-76 | 57-74 (F) | 46 | 4 | |
| 57-80 | 57-79 (F) | 159 | 5 | |
| 57-84 | 57-83 (F) | 150 | 7 | |
| 57-88 | 57-86 | 150 | 7 | Polyethylene tubing scratched out on fifth day |

the protein-depleted dogs, was the comparative absence of edema of the kidney For this we have no explanation at this time

*Discussion*

The lack of increase in survival of the transplanted kidney in the protein-depleted animals was surprising in view of the fact that earlier it had been possible to alter significantly the anaphylactic response in such experimental dogs It should be noted that, just as in the same type of animal in the anaphylaxis experiments,[2] the electrophoretic patterns of the plasma proteins showed increases in the combined $beta_1$- and $beta_2$- globulins and increases rather than expected decreases in the gamma-globulin components The albumin values were markedly lowered in all cases as, of course, were the albumin-globulin (A/G) ratios

In the animals in which arterial injection of the transplanted kidney was made following operation one can hardly be surprised at the lack of effect of the radiation on the kidney The renal tissue contains a relatively small fraction of the total body reticuloendothelial cells, and a kidney takes up less than 0 1 per cent of the total amount injected even by this route These animals show little if any additional concentration of the isotope over and above what would be expected by intravenous administration of the material at some far removed site Therefore, in effect, one is essentially delivering a single relatively large dose of irradiation to the RES, chiefly the liver and spleen, of the recipient

A possibly valid objection to the procedure in which the arterial system of the homotransplanted kidney is injected might be based upon the fact that the antigenic response might be initiated immediately following operation and

preceding the injection of the radioactive material, even though the time lag represented only a matter of a few minutes We cannot account logically at present for the lack of edema in the kidney transplants of these animals

Some preliminary experiments have been carried out in which the intended recipient is injected with amounts of colloidal gold to provide massive irradiation of the RES for 1 or 2 days prior to transplantation of a donor kidney There are 2 disadvantages to this procedure First, if the amount of radiation delivered is much more than 10,000 rads, the recipient would be expected to survive for only 9 to 11 days Therefore, we are limited as to the length of time such irradiation can be allowed to proceed before homotransplantation in order that the survival of the recipient will be sufficiently longer than that of the transplanted kidney to allow for such evaluation The second objection to this approach lies in the fact that the homotransplantation must be carried out in a relatively high radiation field even in spite of attempts to shield the hepatic and splenic areas

Other experiments are in progress in collaboration with J H Muller of the University of Zurich, Switzerland, to circumvent the inability to concentrate the radiation effect in such transplanted kidneys Here, instead of making use of the phagocytic properties of the RES, we rely upon the filtration of large particles by the arterial capillary system in the injected organ as first suggested by the work of Muller and Rossier[13] In this approach carbon particles of about 1000 times the size of the colloidal gold (that is, in the neighborhood of 50 $\mu$) are used Gold chloride is adsorbed onto the carbon, after which it is reduced to metallic gold The material is then irradiated with thermal neutrons It is then suspended in a solution of dextran and injected into the carotid artery supplying the homotransplanted kidney The latter studies are in their preliminary stages and will be discussed elsewhere at a later date

The massive irradiation of the RES in the animals intended as donors is directed at a generalized attack upon the RES on the assumption either that the cells of this system are directly involved in the antigen-antibody reaction or that indirectly the plasma cells derived from such tissue or the decrease in lymphocytes resulting from irradiation of the hematopoietic system might mirror some changes in the immune response Such donor animals do not survive more than a few days after the nephrectomy and are, of course, considered expendable

What interest may attach to the studies described above will hardly be of a practical nature, since the amounts of radiation involved are such as to preclude their use in humans The lowest dose of radiocolloidal gold administered in the cases of arterial injection of new homotransplants is of the order of 10 times those used by us, on a per-kilogram body-weight basis, in successful treatment of chronic leukemias in humans

Our findings in this and other work involving the massive irradiation of the reticuloendothelial system (RES) are certainly not in accord with the statement of Gabrielli[14] to the effect that "the RES is one of the most radiosensitive tissues of the body" This statement would be independent of whether antibodies are involved in their formation by the RES Before irreversible histo-

logical and functional changes in the tissues (that is, liver and spleen) are involved, tremendous amounts of irradiation are well tolerated

*Summary*

Nine dogs were grossly depleted of their storage and circulating plasma proteins in an attempt to restrict antibody formation Kidney homotransplants were made using such depleted dogs as donors and recipients or as recipients only In general, it was found that no significant increase in survival of physiological functioning of the kidney was obtained

In a group of 9 dogs, the intended donor was given a massive single dose of $Au^{198}$ colloid by jugular vein approximately one week prior to use This was a quantity often sufficient to result in the death of such an animal in from 9 to 11 days due to an estimated total delivery of 30,000 to 40,000 equivalent roentgens to the reticuloendothelial system When transplanted to the neck of normal recipients the kidneys of such grossly irradiated animals did not maintain their secretory function appreciably longer than the 3 to 6 days found in the untreated animals

In a group of 11 dogs, kidney homotransplants were carried out from normal mongrel donors to normal mongrel recipients, following which an arterial injection of radioactive colloidal gold was made into the transplanted kidney In such animals there was no consistent increase of the kidney functioning capacity, although there was decidedly less reaction of edema than encountered in untreated animals

*References*

1 HAHN, P F & G H WHIPPLE 1939 J Exptl Med **69**. 315–326
2 HAHN, P F, P BAUGH & H C MENG 1956 Proc Soc Exptl Biol Med **93** 448–450
3 VOISIN, G A. & P F HAHN 1958 Resumé des communications Coll Intern Biol Homogreffes . 10–11 In press
4 DEMPSTER, W J 1953 Brit J Surg **40**. 447
5 SIMONSEN, M 1953 Acta Pathol Microbiol Scand **32**. 36
6 WISSLER, R W, F W FITCH, M F LA VIA & C H GUNDERSON 1957 J Cellular and Comp Physiol Suppl **1**. 265–301
7 TALIAFERRO, W H & L G TALIAFERRO 1951 J Immunol **66** 181
8 HAHN, P F, A H JACKSON, F E STAGGERS, M A. JACKSON & E L CAROTHERS 1956 Am J Roentgenol, Radium Therapy Nuclear Med **75** 1139–1143
9 MARINELLI, L D, E H QUIMBY & G J HINE 1948 Am J Roentgenol Radium Therapy Nuclear Med **59**. 266–281
10 WHEELER, B, M A. JACKSON & P F HAHN 1951 Am J Physiol **166**· 323–327
11 HAHN, P F, M A. JACKSON & H GOLDIE 1951 Science **114**: 303–305
12 HAHN, P F, E L CAROTHERS, A H JACKSON, F E STAGGERS & M A JACKSON 1957 Am J Roentgenol Radium Therapy Nuclear Med **77** 476–485
13 MULLER, J H. & P H ROSSIER 1951 Acta Radiologica **35**: 449–468
14 GABRIELLI, E R 1955 Ann N Y Acad Sci **59**(3). 337–342

# SPECIFIC DESENSITIZATION OF GUINEA PIGS WITH DELAYED HYPERSENSITIVITY TO PROTEIN ANTIGENS*

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A general method for the experimental induction in guinea pigs and in man of the delayed hypersensitive state directed against protein antigens has been described previously.[1, 2] Sensitization follows within a few days after a single intradermal injection of a small amount of antigen in the form of an immune precipitate. Provided that the specific precipitation is carried out in the region of antibody excess, active antibody production is suppressed, and no antibody can be detected in the circulation for at least two weeks after the delayed type of skin reactivity has appeared. The degree of sensitivity is enhanced if the sensitizing dose is incorporated in an adjuvant that contains killed mycobacteria.

In the present study, guinea pigs were sensitized to protein antigens using specific complexes suspended in complete adjuvant. One to two weeks later, when the animals had become highly sensitive, they were injected by various routes with the corresponding antigen. The effect of this parenteral injection upon subsequent skin reactivity was then observed. Provided the dose was sufficient, desensitization was specific and complete.

*Specificity of desensitization.* Groups of animals were sensitized simultaneously to 2 immunologically distinct antigens. TABLE 1 shows the results of a typical experiment in which 6 guinea pigs were sensitized to both ovalbumin and diphtheria toxoid. Seven days after the sensitizing injections, 3 animals received 4.2 mg. ovalbumin intraperitoneally, and the other 3 received 4.2 mg. toxoid. Seven hours later all of the animals were skin-tested for the first time with both ovalbumin and toxoid. As can be seen from TABLE 1, none of the animals showed skin reactions to the antigen that had been used for desensitization, but all reacted strongly to the second antigen against which they had been sensitized. Even as much as 300 μg. ovalbumin failed to elicit skin reactions in 2 guinea pigs after they had been desensitized with 4.2 mg. ovalbumin. Strictly analogous results were obtained in a similar type of experiment in which 6 guinea pigs were sensitized to both ovalbumin and horse gamma-globulin.

None of the experimental animals showed signs of distress at any time after receiving a desensitizing dose of specific antigen. In particular, they showed no sign of acute or protracted anaphylactic shock after intravenous or intraperitoneal challenge, nor were there signs suggestive of the type of delayed shock seen in tuberculous guinea pigs injected with a relatively large amount of tuberculin.

The delayed skin reaction is the most sensitive and, at present, the only reliable criterion available for detecting the delayed hypersensitive state in

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TABLE 1
SPECIFICITY OF DESENSITIZATION

| Desensitizing dose | Skin reactions in mm | |
|---|---|---|
| | 3 μg toxoid | 3 μg ovalbumin |
| 4 2 mg ovalbumin | 24 × 26 | 0* |
| | 16 × 14 | 0* |
| | 20 × 12 | 0 |
| 4 2 mg toxoid | 0 | 20 × 16 |
| | 0 | 17 × 12 |
| | 0 | 19 × 16 |

All animals were sensitized to both ovalbumin and diphtheria toxin 7 days prior to desensitization They were skin-tested with both ovalbumin and toxoid 7 hours after desensitization

* These animals were also skin-tested with 300 μg ovalbumin at the same time, and no skin reactions were elicited

the guinea pig The experiments described above show that, according to this criterion, guinea pigs sensitized to protein antigens can be specifically and completely desensitized by a single injection of the corresponding antigen The ease with which these experimentally sensitized animals could be desensitized contrasts with the difficulties encountered by investigators who have attempted to desensitize tuberculous guinea pigs to tuberculin [8]

*Amount of antigen required for desensitization* Twenty-five animals were sensitized to ovalbumin Nine days later, groups of animals were injected with decreasing amounts of ovalbumin After a further 12 hours all the animals were skin-tested for the first time with 5 μg of ovalbumin Three sensitized control animals showed delayed skin reactions averaging 41 mm in diameter Seven of 8 animals that received 1 8 or more mg ovalbumin parenterally showed no skin reactions on subsequent intradermal challenge The remaining animal showed a 7 × 12 mm reaction When smaller amounts of ovalbumin were used desensitization was progressively less complete However, as little as 18 μg of ovalbumin appeared to decrease the diameter of the "expected" skin reactions The intravenous, intraperitoneal, and intradermal routes were used for desensitization and appeared to be equally effective

*Duration of the desensitized state* In order to determine how long desensitized animals remained nonreactive to specific intradermal challenge, desensitized animals were skin-tested at intervals following the initial negative skin test The duration of the nonreactive period was longer when larger amounts of antigen were used in the desensitizing injections Thus, when 1 8 mg of ovalbumin was administered the desensitized animals showed no skin reactivity for 3 days, if 67 5 mg was used, the animals were nonreactive for at least 1 week Along with or immediately following the return of skin reactivity, small amounts of antibody were detectable in the sera of the animals by passive cutaneous anaphylaxis Reactions of the Arthus type usually could be elicited on the following day

*Desensitization with specific complexes* In these experiments animals were sensitized to diphtheria toxin using toxin–rabbit antitoxin complexes From

TABLE 2
DESENSITIZATION BEFORE AND AFTER SKIN TESTING

| No of animals | Time of desensitization before (−) or after (+) skin testing in hours | Skin reactions in mm 5 μg ovalbumin |
|---|---|---|
| 3 | Not desensitized | 42 × 40, 55 × 40, 70 × 50 |
| 3 | −5 | 0, 0, 0 |
| 3 | 0 | 0, 0, 0 |
| 4 | +1 | 0, 0, 0, 8 × 6 |
| 2 | +5 | 24 × 18, 17 × 19 |

All animals were sensitized to ovalbumin 9 days prior to desensitization with 3 5 mg of ovalbumin administered intravenously

8 to 12 days later the animals were injected intravenously with specific complexes formed with horse antitoxin and were skin-tested with 3 μg of toxoid several hours later Four guinea pigs received 15 Lf or 40 μg of diphtheria toxin, which was mixed with horse antitoxin in slight excess and then immediately injected intravenously before visible aggregation had taken place The average diameter of the skin reactions was 9 mm compared to an average of 29 mm in 5 sensitized control animals Desensitization was complete when 560 Lf (1 4 mg ) of diphtheria toxoid complexed to excess antitoxin was injected into 4 guinea pigs Consequently, it would appear that combination of antigen with slight excess homologous antibody does not alter appreciably its capacity to bring about desensitization

*Desensitization before and after skin testing* In the experiments described thus far, the desensitizing dose of antigen was always administered several hours before skin testing Fifteen guinea pigs were sensitized to ovalbumin Nine days later, 12 animals received 3 5 mg ovalbumin intravenously Groups of these animals were skin-tested with 5 μg ovalbumin at intervals extending from 5 hours prior to the desensitizing dose to 5 hours thereafter As shown in TABLE 2, the intravenous injection of ovalbumin prevented the appearance of skin reactions in 3 of 4 animals even when given 1 hour after the skin test In fact, even when the desensitizing dose was administered as late as 5 hours after skin tests, at a time when delayed reactions were just beginning to appear, the lesions that developed were much smaller than those seen in the control animals

These results help to give some concept of the speed of the immunological events occurring in the delayed skin reaction Up to 1 hour (even longer in other experiments) after the intradermal introduction of the antigen, the interaction between the injected antigen and antigen-specific factor in the tissues has progressed so slowly that its interruption at this time may still prevent the appearance of any macroscopic inflammation

## *Summary*

(1) Guinea pigs with delayed hypersensitivity to protein antigens can be specifically and completely desensitized by a single injection containing a sufficient amount of the corresponding antigen

(2) Desensitization can be accomplished with the antigen-antibody complex, as well as by "free" antigen

(3) The appearance of the delayed skin reaction can be prevented in fully sensitized animals even when a desensitizing dose is given 1 hour or more *after* skin testing

### *References*

1 UHR, J W, S B SALVIN & A M PAPPENHEIMER, JR 1957 Production of delayed sensitivity to protein without detectable circulating antibody Ann N Y Acad Sci **64**(5): 877

2 UHR, J W, S B SALVIN & A M PAPPENHEIMER, JR 1957 Delayed hypersensitivity II Induction of hypersensitivity in guinea pigs by means of antigen-antibody complexes J Exptl Med **105** 11

3 RICH, A R 1951 The Pathogenesis of Tuberculosis 2nd ed : 436–450 Thomas Springfield, Ill

# ANTIGENS RESPONSIBLE FOR BONE MARROW TRANSPLANTATION IMMUNITY

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It is now established that transplantation of foreign bone marrow can take place in X-irradiated recipients,[1-7] and that X-radiation death can be prevented by treatment with isologous,‡ homologous, or closely related heterologous bone marrow [8-11] However, lethally X-irradiated mice receiving foreign bone marrow show, in general, a characteristic delayed reaction culminating in a high percentage of death 30 to 60 days after the treatment [2, 12-14] Our conclusion, based on morphologic, electrophoretic, and immunologic changes,[2, 15-20] was that the "foreign bone marrow reaction" is caused by an *in vivo* reaction between antigen and antibody We further deduced that this reaction involved the host-immune mechanism, which was recovering from the radiation effect, and the antigens of the proliferating foreign bone marrow transplant Obviously, if the host were sensitized to the foreign bone marrow antigens prior to the radiation and bone marrow treatment, an earlier and greater percentage of death would be expected This has been found to be the case [21]

Billingham *et al* have demonstrated that the antigenic materials responsible for skin homograft immunity in mice are labile desoxyribonucleoproteinlike substances [22] Since radiation-induced bone marrow chimeras can also accept skin grafts of genetic constitution comparable to the bone marrow,[23-25] it would be interesting to characterize the antigens responsible for bone marrow transplantation immunity This report describes the localization and nature of such antigens

## *Materials and Methods*

*Animals* Normal (C3H × 101) $F_1$ male and female mice were used as hosts in the following experiments They were caged in groups of 5 to 10 and allowed free access to food and water Normal male and female Sprague-Dawley rats weighing 150 to 200 gm were used as donors

*Antigens* At 6 or 10 weeks of age mice were sensitized by the intravenous or intraperitoneal route with various antigens Bone marrow cells (BMC) from rat femurs were suspended in Tyrode's solution The red blood cell (RBC) suspension was prepared by centrifuging whole blood, carefully removing the buffy layer, and washing the RBC 3 times with 50 volumes of 0 15 M NaCl White blood cells (WBC) were separated from the RBC in a 6 per cent fibrinogen solution, an RBC-agglomerating agent [26 27] They were washed

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‡ Isologous denotes members of the same strain within a species, homologous denotes members of different strains within a species, and heterologous denotes members of different species

3 times and suspended as above The concentration of these antigens and of the liver nuclei (see next paragraph) was $1 \times 10^6$ per ml

For the nuclear, mitochondrial, and microsomal fractions, liver brei was subjected to a density-gradient centrifugation according to the Albright-Anderson method[28] The liver brei supernatant fraction was prepared according to the method of Anderson,[29] and desoxyribonucleoprotein (DNA protein) was prepared from liver nuclei as described by Mirsky and Pollister[30] Whole rat serum (RS) was obtained from a pool of normal rat sera The concentration of these antigens, except RS, was 2 mg of Kjeldahl nitrogen per ml

Crystalline desoxyribonuclease (DNase), ribonuclease (RNase), trypsin,* and papain powder, kindly prepared by D G Doherty of this laboratory, were used in the treatment of some of these antigens The enzyme concentration was 0 1 mg /ml of antigen solution Incubation was carried out at room temperature for 30 minutes Phase microscopic observation was made of antigens before and after the enzyme treatment

*X irradiation* A constant-potential Phillips X-ray machine was used as the source, with the following conditions 250 kv , 15 mAmp , 160 r/min at 60 cm , inherent filtration, 1 0 mm of Al, added filtration, 1 0 mm of Al, and HVL, 0 5 mm of Cu At 12 weeks of age the mice were placed in a circular, perforated Lucite container attached to a revolving turntable and given a single dose of 950 r of X radiation

*Bone marrow treatment* Within 4 hours after irradiation each mouse, except the radiation control mice, received intravenously $140 \times 10^6$ nucleated BMC obtained from fresh rat femurs

The mice were inspected daily throughout the investigation No deaths occurred within the first 3 days after the 950 r and BMC treatment (950 r-RBM), indicating that there was no immediate fatal anaphylaxis

## *Results*

*Localization of sensitizing antigens* The dose per mouse of RBC, WBC, BMC, and nuclei was $1 \times 10^6$, of DNA-protein, mitochondria, microsomes, supernatant, and RS, 0 6 mg of nitrogen Fifteen per cent of the nonsensitized mice died by the fifteenth day after 950 r-RBM, and 22 per cent were dead by the thirtieth day Death patterns of mice sensitized to RBC, RS, or supernatant were comparable to those of the nonsensitized mice All animals sensitized to WBC or BMC died within 15 days after 950 r-RBM Mortality of mice sensitized to nuclei was 68 per cent 15 days after 950 r-RBM and was 70 per cent by the thirtieth day Mortality was also high among mice sensitized to mitochondrial and microsomal antigens, the 15- and 30-day mortality of those sensitized to mitochondria was 80 and 85 per cent, respectively, and of those sensitized to microsomes, 65 and 85 per cent, respectively These results are shown in TABLE 1

*Dose effect* That the sensitizing effect of these antigens is dose dependent is evident from results shown in TABLE 2 Increasing the dose of nuclei from $1 \times 10^5$ to $1 \times 10^6$ to $2 \times 10^6$ per mouse increased the mortality proportion-

* Obtained from the Worthington Biological Sales Co , New York, N Y

TABLE 1
LOCALIZATION OF TRANSPLANTATION ANTIGENS IN RAT CELLS

| Sensitizing antigens* | No of mice | Mortality after 950 r-RBM (%) | |
|---|---|---|---|
| | | 15 days | 30 days |
| None | 27 | 15 | 22 |
| RBC | 30 | 13 | 27 |
| BMC | 30 | 100 | — |
| WBC | 10 | 100 | — |
| Liver cell fractions | | | |
| Nuclei | 40 | 68 | 70 |
| DNA-protein | 20 | 75 | 75 |
| Mitochondria | 20 | 80 | 85 |
| Microsomes | 20 | 65 | 85 |
| Supernatant | 20 | 10 | 20 |
| Serum | 20 | 5 | 15 |

* Dose per mouse of RBC, BMC, WBC, and nuclei was $1 \times 10^6$, and of DNA-protein, mitochondria, microsomes, supernatant, and whole serum 0 6 mg of nitrogen

TABLE 2
DOSE EFFECT OF TRANSPLANTATION ANTIGENS

| Sensitizing antigens | Dose per mouse | No of mice | Mortality after 950 r-RBM (%) | |
|---|---|---|---|---|
| | | | 15 days | 30 days |
| BMC | $1 \times 10^5$ | 10 | 50 | 80 |
| | $1 \times 10^6$ | 30 | 100 | — |
| WBC | $1 \times 10^5$ | 10 | 40 | 60 |
| | $1 \times 10^6$ | 10 | 100 | — |
| Liver nuclei | $1 \times 10^5$ | 40 | 12 | 15 |
| | $1 \times 10^6$ | 40 | 68 | 70 |
| | $2 \times 10^6$ | 20 | 100 | — |
| Liver supernatant | 0 6 mg of N | 20 | 15 | 30 |
| | 3 0 mg of N | 16 | 69 | 81 |
| Whole serum | 0 6 mg of N | 20 | 5 | 15 |
| | 6 5 mg of N | 20 | 10 | 25 |

ately from 12 to 68 to 90 per cent 15 days after 950 r-RBM The mortality curve for mice sensitized to BMC and WBC was similarly dose dependent A supernatant dose of 3 0 mg of nitrogen/ml has a sensitizing effect, but no antigenic activity was detected with a dose of 0 6 mg of nitrogen per mouse Presensitizing activity was not detected in RS in spite of a tenfold increase in the antigen concentration to 6 5 mg of nitrogen per mouse

*Enzyme treatment* The dose of nuclei was $1 \times 10^5$, $1 \times 10^6$, or $2 \times 10^6$ per mouse, the dose of mitochondria, microsomes, and supernatant was 0 6 mg of nitrogen Phase microscopic observations were made of nuclei before and after enzyme treatment After trypsin and papain treatments, the nuclei were severely damaged, with only a few identifiable nuclear "ghosts" After DNase treatment the nuclei still maintained their discrete spherical envelopes, but chromatin precipitation was evident and the nucleoli were poorly defined

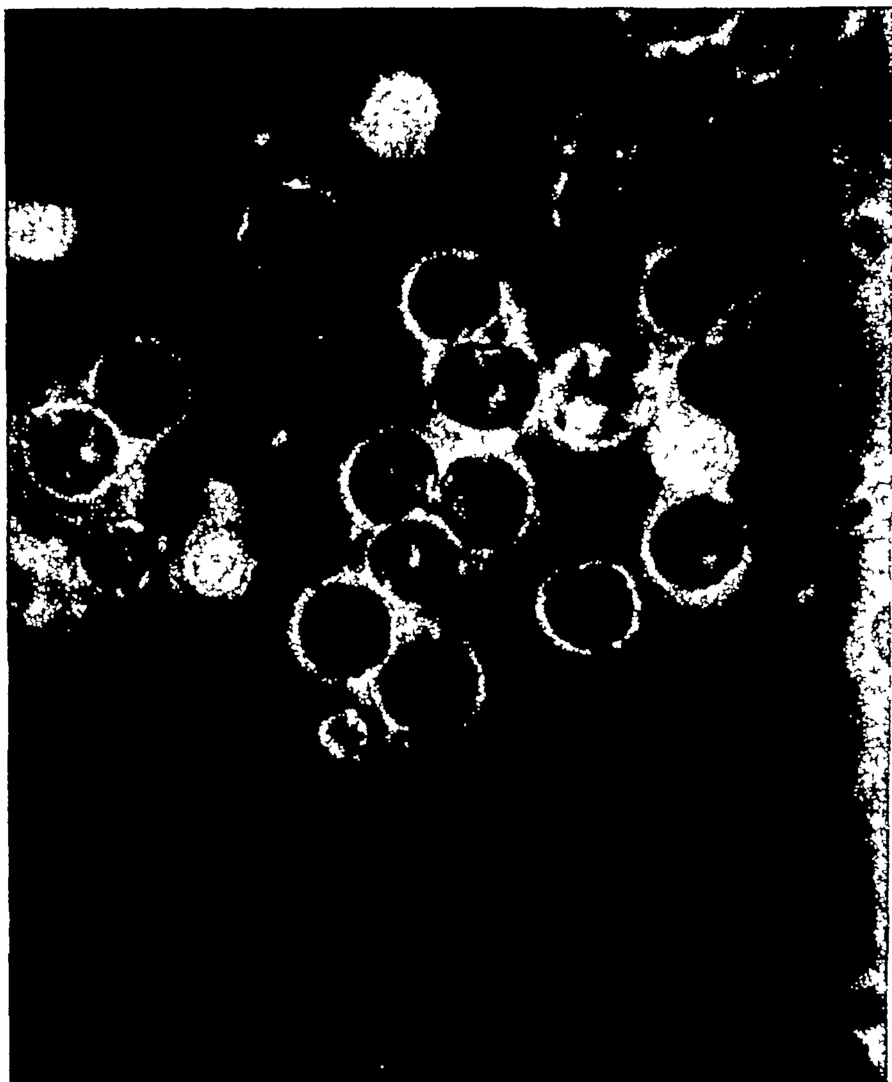


FIGURE 1 Phase contrast photomicrograph of normal untreated nucleus × 1000

After RNase treatment the nuclear envelopes were wrinkled, and there were numerous foci of precipitated chromatin within the nuclei Representative findings are shown in FIGURES 1, 2, and 3

The results of these experiments are tabulated in TABLE 3 Survival data of sensitized 950 r-RBM mice show that the mitochondria and microsomes maintained their sensitizing effect even after treatment with the nucleases and proteases The supernatant fraction at this dose level had no sensitizing effect regardless of the enzyme treatment RNase or papain treatment did not decrease the sensitizing effect of nuclei, but trypsin treatment caused a slight decrease The antigenicity of DNase-treated nuclei, however, was variable In one experiment, where the dose was 2 × $10^6$ nuclei per mouse, treatment with DNase did not decrease the antigenicity In the second experiment, where the dose was 1 × $10^6$ nuclei per mouse, a slight but definite decrease in the antigenicity was seen after the DNase treatment

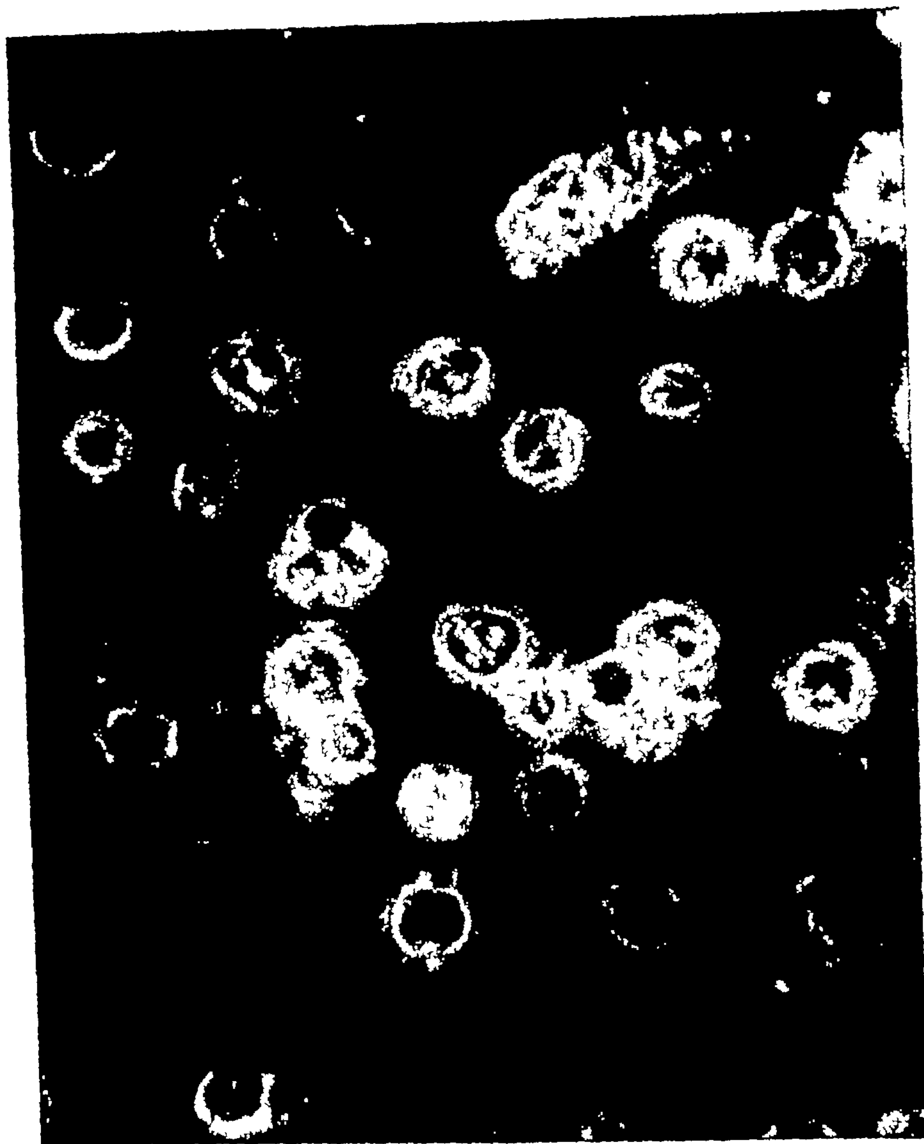


FIGURE 2 Phase contrast photomicrograph of nucleus after treatment with papain × 1000

*Discussion*

The results presented here confirm and extend our preliminary findings[21] The antigens responsible for bone marrow transplantation immunity were shown to reside in nucleated cells and to be absent from RBC and serum To ensure a reasonably "clean" preparation of cell fractions, a freshly perfused liver was used instead of bone marrow, thymus, spleen, or lymph node Furthermore, the newly developed, rapid density gradient centrifugation method of Albright and Anderson[23] was adopted Antigens were localized not only in the nuclear fraction, but also in the mitochondrial and microsomal fractions in a reasonable concentration, and in the supernatant in a relatively low concentration Whether the antigens of the supernatant normally exist in the soluble phase of the cell cannot be ascertained at present These antigens could have



FIGURE 1 Phase contrast photomicrograph of normal untreated nucleus × 1000

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TABLE 3

ENZYME TREATMENT OF TRANSPLANTATION ANTIGENS

| Sensitizing antigens | Dose per mouse | Enzyme | No of mice | Mortality after 950 r-RBM (%) | |
|---|---|---|---|---|---|
| | | | | 15 days | 30 days |
| Supernatant | 0 6 mg of N | None | 20 | 10 | 20 |
| | | DNase | 20 | 0 | 35 |
| | | RNase | 20 | 5 | 15 |
| | | Trypsin | 20 | 5 | 10 |
| Microsomes | 0 6 mg of N | None | 20 | 65 | 85 |
| | | DNase | 20 | 90 | 100 |
| | | RNase | 20 | 65 | 65 |
| | | Trypsin | 20 | 85 | 85 |
| Mitochondria | 0 6 mg of N | None | 20 | 80 | 85 |
| | | RNase | 20 | 60 | 80 |
| | | Trypsin | 20 | 65 | 85 |
| Nuclei | $1 \times 10^5$ | None | 40 | 12 | 15 |
| | | DNase | 20 | 25 | 25 |
| Nuclei | $1 \times 10^6$ | None | 40 | 68 | 70 |
| | | DNase | 20 | 20 | 40 |
| Nuclei | $2 \times 10^6$ | None | 20 | 100 | — |
| | | DNase | 20 | 95 | 95 |
| | | RNase | 20 | 90 | 95 |
| | | Papain | 19 | 100 | — |
| | | Trypsin | 19 | 63 | 84 |

after DNase treatment was variable One preparation of nuclei was slightly susceptible to DNase treatment, another was resistant The DNA-protein materials fractionated from the third preparation of nuclei were also active in their sensitizing effect These results suggest that the antigens residing in the nucleus are labile DNA-proteinlike materials Billingham *et al* [22] suggested that the antigenic materials responsible for skin homograft immunity in mice are also labile DNA-proteinlike substances

Much work must be done before the nature of these antigens for heterologous bone marrow transplantation immunity can be elucidated Furthermore, it is not yet known whether these antigens function in the homologous bone marrow transplantation immunity system Such studies are now being undertaken in this laboratory

*Summary*

(1) The antigens responsible for the bone marrow transplantation immunity were determined by sensitizing the mice 2 or 6 weeks prior to the 950 r and rat bone marrow transplantation (950 r-RBM) Indicative of the sensitizing antigens was the increased mortality 15 and 30 days after 950 r-RBM in comparison to the nonsensitized 950 r-RBM mice

(2) Sensitizing antigens were absent in the serum and red blood cells, but present in nucleated cells They were localized in the nucleus, mitochondria, and microsomes in a reasonable concentration and in the supernatant fraction in a relatively low concentration

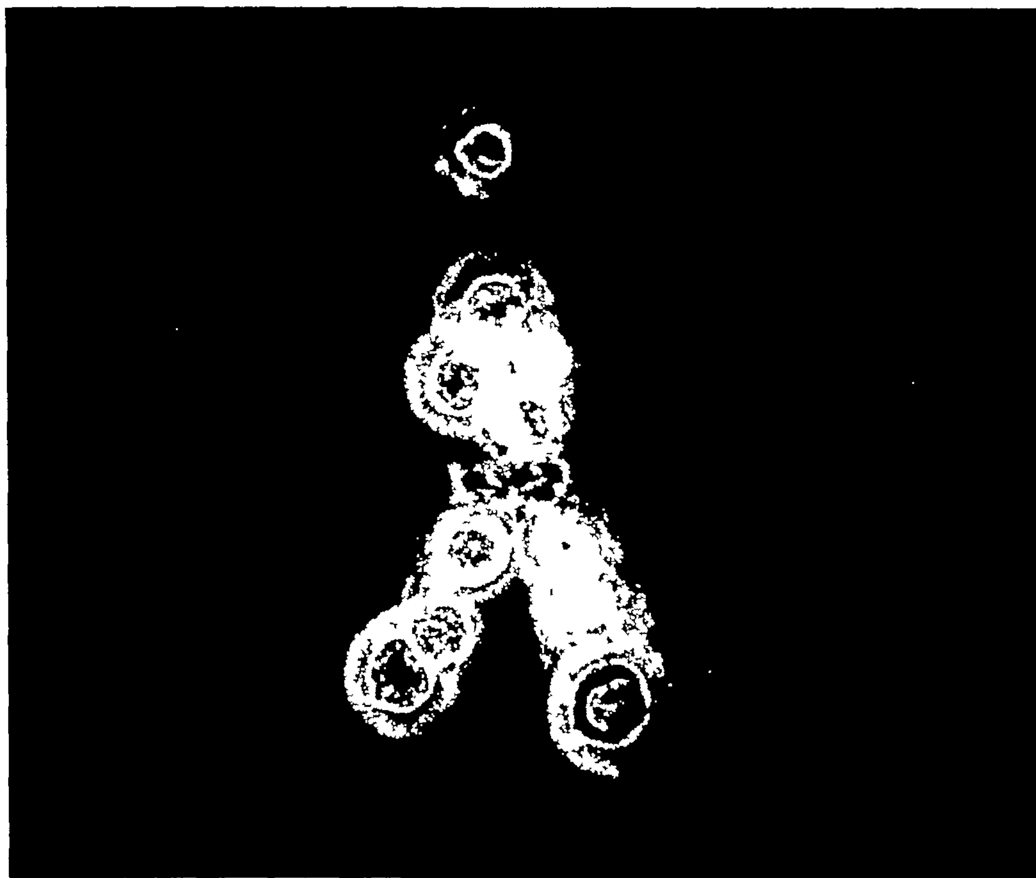


FIGURE 3 Phase contrast photomicrograph of nucleus after treatment with DNase × 1000

"leaked" from the mitochondrial and microsomal bodies during the homogenization

Our results, obtained after various enzyme treatments of cell fractions, indicate the presence of no fewer than two classes of antigens responsible for bone marrow transplantation immunity One class of antigens is localized in the mitochondrial and microsomal fractions, which were resistant to nuclease and protease treatment These findings suggest that the antigens are either simple or complex polysaccharidelike materials or nucleoproteinlike or proteinlike materials encased in a substance resistant to protease and nuclease treatment When the dose of antigens used is taken into account, however, the former possibility seems more likely, that is, the antigens are simple or complex polysaccharidelike materials Past experiences have established the fact that a single injection of antigens in saline in concentration of only 0 6 mg of nitrogen is not adequate if the antigens are proteins[31] or nucleoproteins [32-34] If the antigens are polysaccharidelike materials, however, such a concentration is considered adequate [31]

The second class of antigens is localized in the nucleus and is resistant to RNase and papain, but is slightly susceptible to trypsin The antigenicity

22 BILLINGHAM, R E, L BRENT & P B MEDAWAR 1956 The antigenic stimulus in transplantation immunity Nature 178. 514–519
23 MAIN, J M & R T PREHN 1955 Successful skin homografts after the administration of high dosage X-radiation and homologous bone marrow J Natl Cancer Inst. 15. 1023–1029
24 TRENTIN, J J 1956 Mortality and skin transplantation in X-irradiated mice receiving isologous, homologous and heterologous bone marrow Proc Soc Exptl Biol Med 92. 688–693
25 BROCADES ZAALBERG, O, O VOS & D W VAN BEKKUM 1957 Surviving rat skin grafts in mice Nature 180. 238–239
26 SMITH, L H & C C CONGDON 1957 Leukemoid blood and leukocytes in treatment of radiation lethality A.M A Arch Pathol 63. 502–507
27 SKOOG, W A. & W S BECK 1955 The technics of leukocyte isolation USAEC Unclassified Rept UCLA-333 · 1–46
28 ALBRIGHT, J F & N G ANDERSON 1958 A method for rapid fractionation of particulate systems by gradient differential centrifugation Exptl. Cell Research In press
29 ANDERSON, N G 1956 Techniques for the mass isolation of cellular components *In* Physical Techniques in Biological Research 3. 300–352 G Oster & A W Pollister, Eds Academic Press New York, N Y
30 MIRSKY, A E & A W POLLISTER 1946 Chromosin, a desoxyribosenucleoprotein complex of the cell nucleus J Gen Physiol 30 117–147
31 KABAT, E A & M M MAYER 1948 Experimental Immunochemistry Thomas Springfield, Ill
32 MACULLA, E 1948 The immunochemistry of mouse tissue components I Comparative antigenic composition of normal mouse tissues Yale J Biol Med 20 299–314
33 BLIX, U, C N ILAND & M STACEY 1954 The serological activity of deoxypentose-nucleic acids Brit J Exptl. Pathol 35 241–251
34 HORN, E C 1955 Ascites tumor development I An analysis of the *in vivo* effect of nucleoprotein from Ehrlich ascites cells Cancer Research 15 663–670

## *Discussion of the Paper*

JOHN J TRENTIN (*Baylor University College of Medicine, Houston, Texas*) The early mortality observed in presensitized mice given lethal irradiation followed by rat bone marrow would appear to be consistent also with primary failure of successful heterotransplantation of rat marrow as a result of preformed antibodies Does it necessarily follow, therefore, that the late mortality in nonpresensitized mice protected against lethal irradiation with rat marrow is also the result of an immunological reaction of host against graft? If so, how can one rationalize such an explanation with the absence of secondary hematopoietic depression in mice dead of characteristic late mortality, and with the occurrence of such mortality in some combinations of $F_1$ hybrid mice protected with parent strain marrow where the host, by genetic determination, has no capacity to reject tissue grafts from the homologous marrow donor?

T MAKINODAN There are several known phenomena that are not consistent with your assumptions First, an assumption that rat bone marrow transplantation fails as a result of preformed antibodies is not correct There is direct evidence for the establishment and persistence of the transplant, both in the presensitized mouse-rat bone marrow experiments and in mice presensitized to homologous liver fractions and subsequently treated with radiation and homologous bone marrow (Owen *et al*, unpublished data) Second, the specific secondary antibody response is characteristically quicker and more extreme, and one would therefore expect the preimmunized host's reaction to occur earlier and more vigorously It is difficult to conceive a similar effect of host preimmunization on the graft you postulate Third, it has been reported that

(3) The antigens of the mitochondria and microsomes were resistant to nuclease and protease treatment The nuclear antigens were resistant to ribonuclease and papain treatment, and slightly susceptible to trypsin The antigenicity of desoxyribonuclease-treated nuclei was variable

*References*


1 LINDSLEY, D L, T. T ODELL & F G TAUSCHE 1956 Implantation of functional erythropoietic elements following total-body irradiation Proc Soc Exptl Biol. Med **90.** 512–515

2 MAKINODAN, T 1956 Circulating rat cells in lethally irradiated mice protected with rat bone marrow Proc Soc Exptl Biol Med **92:** 174–179

3 NOWELL, P C, L J COLE, J G HABERMEYER & P L ROAN 1956 Growth and continued function of rat marrow cells in X-irradiated mice Cancer Research **16** 258–261

4 VOS, O, J A G DAVIDS, W W H WEYZEN & D W VAN BEKKUM 1956 Evidence for the cellular hypothesis in radiation protection by bone marrow cells Acta Physiol Pharmacol Neerl **4** 482–486

5 FORD, C E, J L HAMERTON, D W H BARNES & J F LOUTIT 1956 Cytological identification of radiation-chimaeras Nature **177**· 452–454

6 SMITH, L H, T MAKINODAN & C C CONGDON 1957 Circulating rat platelets in lethally X-irradiated mice given rat bone marrow Cancer Research **17** 367–369

7 GENGOZIAN, N, I S URSO, C C CONGDON, A D CONGER & T MAKINODAN 1957 Thymus specificity in lethally irradiated mice treated with rat bone marrow Proc Soc Exptl Biol Med **96**· 714–720

8 JACOBSON, L O, E K MARKS, E O GASTON, M ROBSON & R E ZIRKLE 1949 Role of the spleen in radiation injury Proc Soc Exptl Biol Med **70.** 740–742

9 LORENZ, E, D UPHOFF, T R REID & E SHELTON 1951 Modification of irradiation injury in mice and guinea pigs by bone marrow injections J Natl Cancer Inst. **12** 197–201

10 COLE, L J, M C FISHLER, M E ELLIS & V P BOND 1952 Protection of mice against X-irradiation by spleen homogenates administered after exposure Proc Soc Exptl Biol Med **80**· 112–117

11 LORENZ, E & C C CONGDON 1952 Modification of acute and chronic irradiation injury in experimental animals by injection of bone marrow and tissues originating from hematopoietic organs Proc Intern Congr Intern Soc Hematol **4.** 192–211

12 CONGDON, C C & E LORENZ 1954 Humoral factor in irradiation protection modification of lethal irradiation injury in mice by injection of rat bone marrow Am J Physiol **176** 297–300

13 BARNES, D W H & J F LOUTIT 1954 What is the recovery factor in spleen? Nucleonics **12**(5) 68–71

14 COLE, L J, J G HABERMEYER & V P BOND 1955 Recovery from acute radiation injury in mice following administration of rat bone marrow J Natl Cancer Inst **16** 1–9

15 MAKINODAN, T, N GENGOZIAN & C C CONGDON 1956 Agglutinin production in normal, sublethally irradiated, and lethally irradiated mice treated with mouse bone marrow J Immunol **77**· 250–256

16 GENGOZIAN, N & T MAKINODAN 1956 Antibody response of lethally X-irradiated mice treated with rat bone marrow J Immunol **77** 430–436

17 SHEKARCHI, I C & T MAKINODAN 1957 Electrophoretic studies on the sera of mice treated with 950 r of X rays and rat bone marrow Radiation Research **7:** 449

18 CONGDON, C C, T MAKINODAN & N GENGOZIAN 1957 Effect of injection of rat bone marrow on reticular tissues of mice exposed to X-radiation in midlethal dose range. J Natl Cancer Inst **18** 603–613

19 MAKINODAN, T & N G ANDERSON 1957 Physicochemical properties of circulating red blood cells of lethally X-irradiated mice treated with rat bone marrow Blood **12** 984–992

20 GENGOZIAN, N & T MAKINODAN 1957 Mortality of mice as affected by variation of the X-ray dose and number of nucleated rat bone marrow cells injected Cancer Research **17** 970–975

21 MAKINODAN, T 1957 Immunology of bone marrow transplantation J Cellular Comp Physiol Suppl 1 **50** 327–351

# THE ERYTHROCYTE-BORNE ANTIGEN IN TUMOR IMMUNITY

*By* Morris K Barrett
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In 1908 Bashford *et al* reported[1] that resistance to a graft of transplantable tumor could be induced by a prior inoculation of neoplastic or normal tissue Defibrinated blood was one of the normal tissues used by these investigators During the past fifty years this observation has been confirmed many times, and several bits of additional information have been discovered by contributors too numerous for mention here, references to their work can be found in recent reviews [2-4]

Although there is not complete accord, the generally held view has been that this antigenic* property is widely distributed, if not ubiquitous, among living cells Recent findings have forced some modification of the requirement that the cells must be living or intact [5, 6] It is important to know whether the erythrocyte shares this property because, if such antigenicity is an attribute of the red cell membrane, opportunities seem to exist for obtaining a relatively simple starting material for further work, and there may be important theoretical implications regarding not only transplantation immunity but also the cellular interactions in differentiation and growth The idea involved is similar to Weiss's concept of "molecular ecology,"[7] but we are thinking of a possible "cellular ecology" that may or may not be limited to the molecular level

Previous data[5, 6] supported the belief that the red cell membrane does, indeed, carry such an antigen and suggested some tentative ideas regarding its nature In the following paragraphs the evidence is briefly reviewed in a manner giving prominence to certain points not previously emphasized, and new evidence is added

Using a strain DBA/2 sarcoma that grows progressively in 90 per cent of strain BALB/c mice, we found that a high degree of resistance could be induced in BALB/c hosts by a prior injection of defibrinated blood from strain DBA/2 Experiments involving separation of serum and cells showed that the antigen was in the cells and not in the serum, as Bashford had also observed Separation of red cells from white cells and use of each as antigen indicated that both fractions were antigenic, as expected, however, when the dose was expressed in terms of the original volume of blood represented, the red cell fraction was more potent than the white cell fraction, probably because of numerical relations TABLE 1 shows aggregated data from three such experiments One feature of these data should be noted particularly All the white cells that could be recovered (by the method used) from 0 2 cc of blood produced resistance at about the 50 per cent level Although the experiments might have been more refined, this figure can be taken as a useful approximation in relation to data to be given later An investigation of the effect of dosage, always expressed as equivalent blood volume, was made using

* The term immunity is used here to refer to a host's resistance to the implantation of a graft The term antigen refers to any substance that will induce such immunity

the histological changes observed at intervals after irradiation-foreign bone marrow treatment are qualitatively comparable and differ only quantitatively between early deaths and late deaths Since the kinetic aspects of these reactions are different, one cannot expect comparable quantitative histology at the time of death between mice dying 30 to 60 days after treatment and those dying within 30 days Finally, the possibility that the one dominant gene-one antigen concept need not be correct in the irradiation-bone marrow study is discussed by Fox in this monograph and, on other grounds, by Owen independently [1] Therefore, to assume that the $F_1$ host has no capacity to react against parental marrow is not necessarily valid

*Reference*

1 OWEN, RAY D Genetic aspects of tissue transplantation and tolerance Am J Human Genet In press

# THE ERYTHROCYTE-BORNE ANTIGEN IN TUMOR IMMUNITY

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* The term immunity is used here to refer to a host's resistance to the implantation of a graft The term antigen refers to any substance that will induce such immunity

TABLE 1

| Inocula | Defib blood | RBC | WBC | Serum |
|---|---|---|---|---|
| Per cent | 84 | 78 | 52 | 15 |
| Total hosts | (57) | (50) | (52) | (50) |

Degree of resistance to implantation of tumor induced in strain BALB/c hosts by a prior inoculation of a fraction of strain DBA/2 blood All doses were equivalent to 0 2 cc of defibrinated blood Results are shown as percentage of hosts immune Number of hosts is shown in parentheses

both defibrinated blood and washed cell suspensions The expected graded effect of dose was seen, and it was indicated that a response at the 50 per cent level would be expected with doses of between 0 003 and 0 006 cc, or about 1/40 of the previous dose For most subsequent experiments we selected a dose of 0 02 cc that produces a strong but not maximal effect It should be noted that this dose is 1/10 of that used for white cells At this dose washed cells or stromata immunized about 80 per cent of hosts, that is, 20 per cent had tumors, as shown in TABLE 2

When the relative numbers of cells present in the inocula are considered, the data of TABLES 1 and 2 suggest that, in equal numbers, the white cells are probably a more potent antigen than the red cells, but that is not the point under consideration Our experiments were directed at the question, "Is the erythrocyte stroma antigenic?" At present we are not seeking its relative potency

The data of TABLE 2 are based on a dose of 0 02 cc In many experiments involving various cell preparations we have observed this degree of response to this dose despite the fact that the proportion of white cells contained in the inocula varied widely Several regular chamber counts on our preparations indicated that defibrination reduced the white count by about 1/2, multiple washing reduced it to about 1/5, sometimes 1/10 Sometimes a few white cells were seen in the various preparations of stromata, but in others none were found Yet defibrinated blood, washed cells, and stromata all produced similar degrees of resistance, that is, about 20 per cent tumors in immune animals compared with 90 per cent in controls In the same inocula the red cell count remained high, varying from 75 to 100 per cent of that in defibrinated blood, and depending on the amount of handling involved

TABLE 2

| Antigen | Mice | Tumors | Per cent immune |
|---|---|---|---|
| None | 60 | 56 | 7 |
| Washed RBC | 158 | 30 | 81 |
| Stromata prepared with | | | |
| Saline | 129 | 26 | 80 |
| Citrate | 30 | 4 | 87 |
| Antiserum | 60 | 7 | 88 |

Resistance to implantation of strain DBA tumor in strain BALB/c mice after immunization by RBC or stromata from strain DBA blood All doses were equivalent to 0 02 cc of blood

Thus it appears that the immune response varies with the dose of blood, but for a given dose of blood the effect does not vary significantly with a decreasing content of white cells if the red cell count remains high We thought that this was sufficient evidence to justify a belief that in our work the observed effect depended upon some attribute of the red cell and not upon the few white cells contaminating some of our inocula For that reason these points were mentioned only briefly in previous discussions

However, the point has again been raised by Billingham *et al*[8-10] Their work concerns transplantation of skin, whereas ours concerns transplantation of tumors This distinction may be critical, although there are other differences in technique If this distinction is critical, it would be new as a generalization and quite important However, these investigators, like most workers in this field, link resistance to grafts of normal tissue with that to grafts of tumor under the general term "transplantation immunity" We therefore adopted one of their techniques for carrying our observations a little farther Glass wool filters were prepared in the manner described by Billingham[9] and used to remove white cells from cell suspensions Thrice-washed cell suspensions were prepared as before and, following filtration, were used to induce tumor resistance[5, 6] Two experiments were done, in each a white cell count was done on unfiltered washed cells In the first the count was 350 and in the second, 440/mm $^3$, representing about $\frac{1}{10}$ of the count on defibrinated blood in this strain Each cell suspension was then filtered thrice successively through each of 3 filters (1 more than was used by Billingham) Chamber counts and stained smears were made for each suspension, no white cells were found in either smear, but among 4 chambers (2 for each suspension) a single white cell was seen in 1 chamber (representing 25/mm $^3$, by our technique, for 1 suspension and none for the other) Both suspensions were strongly antigenic in doses of 0 02 cc , as shown in TABLE 3 (last 2 lines)

Recalling again that white cells from 10 times this much blood did not induce this degree of immunity (TABLE 1), but that the same degree persisted when the white count was progressively reduced by more than tenfold, it seems that one must conclude that the antigen with which we are working is present in the red cell fraction This is not to say that the white cell does not also carry an antigen, that is another matter

Turning to the question of whether some of the discordances in this field are dependent upon differences between normal and neoplastic tissues we have made some preliminary observations on the power of red cell suspensions to induce immunity to normal tissue It is generally recognized that tumors are more easily transplanted than are normal tissues, and that some variance between observations depends upon this difference We have adopted the viewpoint that, for the present, it is better to look upon such differences as being quantitative in nature The point is important because, in our opinion, the establishment of criteria for a truly qualitative difference between normal and neoplastic tissue would be a discovery of the first magnitude

Merwin has reported observations on immunity reactions in nonvascularized homologous grafts of normal Harderian gland[11] Such grafts will survive indefinitely in nonimmune homologous hosts, but they disintegrate if the hosts are

TABLE 3

| Inocula | Equiv dose, cc | Number of cells | | Number of hosts | Per cent immune |
|---|---|---|---|---|---|
| | | RBC | WBC | | |
| Defib blood | 0 2 | 1,400,000,000 | 600,000 | 50 | 94 |
| Defib blood | 0 025 | 175,000,000 | 75,000 | 40 | 92 |
| Defib blood | 0 003 | 21,000,000 | 9,000 | 25 | 40 |
| Defib blood | 0 002 | 14,000,000 | 6,000 | 24 | 54* |
| Washed cells | 0 2 | 1,200,000,000 | 60,000 | 54 | 94 |
| Washed cells | 0 02 | 120,000,000 | 10–20,000 | 158 | 81 |
| Isolated cells | 0 2 | 1,400,000,000 | few | 50 | 78† |
| Isolated cells | 0 2 | few | 600,000 | 52 | 52† |
| Stromata | 0 02 | 120,000,000 | 0–very few | 219 | 83 |
| Filtered cells | 0 02 | 120,000,000 | 0 (?) | 30 | 97 |
| Filtered cells | 0 02 | 120,000,000 | 500 (?) | 49 | 78 |

Comparison of degree of resistance induced by various fractions and doses of blood with particular reference to the relative numbers of red cells and white cells contained in the inocula Cell numbers are approximate and calculated from standard chamber counts on varied preparations and from the doses used Data are taken from various published and unpublished experiments Equivalent doses refer to volume of defibrinated blood represented by inocula

* Data from R T Prehn (personal communication)

† Procedure involved prolonged handling, and cells were probably damaged to a minor degree The difference between this pair is highly significant (P = 0 008)

immunized by another graft In cooperation with her we have experiments in progress to ascertain the effect of inoculation of homologous red cell suspensions upon established nonvascularized grafts of homologous Harderian glands in mice

Filtered blood was prepared as before, and the filtrate was examined for white cells For unexplained reasons filtration was not quite as effective as before, even so, the proportion of white cells was always quite small, sometimes only 1 cell in 300 high-power fields of a stained smear Cell suspensions were used in doses of either 0 2 or 0 02 cc equivalence of defibrinated blood, inoculated subcutaneously remote from the graft Immunity (that is, graft destruction) was observed after both doses The majority of grafts broke down after the larger dose and, judged on the basis of Merwin's previous experience,[11] the effect appeared to be stronger than could be accounted for by the small number of white cells present These must be viewed as preliminary results and subject to revision, but they seem to indicate that the antigen borne by the erythrocyte is capable of inducing resistance against normal homologous Harderian gland It is expected that more evidence on this point will emerge from future experiments

Elsewhere I have mentioned Breyere's observation that the survival time of skin homografts on pen-bred rats was shortened when the recipient had received a prior inoculation of the donor's blood [12] The results were similar in inbred rats, but they were more uniform because of the employment of inbred animals In this case it is difficult to evaluate the relative influence of white cells in the absence of data on graded doses, but one is encouraged to

look further for a possible influence of the red cell Here, too, more work needs to be done before reaching conclusions

In summation, the data related to tumor immunity seem to point clearly to the conclusion that there is an effective antigen on the erythrocyte stroma In our case it is not controlled by the histocompatibility-2 locus of Snell *et al*[13] because all our materials are H-$2^d$, but in other cases this may be an important influence In the case of normal tissues the data are not so clear, and this distinction may be more important than one would assume at present No attempt has been made to assess the relative potency of the erythrocyte-borne antigen because, in our opinion, the important point is that it is there

*References*


1 BASHFORD, E , J A MURRAY & W CRAMER 1908 The natural and induced resistance of mice to the growth of cancer 3rd Sci Rept Brit Emp Cancer Research Fund : 315–340

2 HAUSCHKA, T S 1952 Immunological aspects of cancer a review Cancer Research **12** 615–633

3 SNELL, G D 1953 Transplantable tumors *In* The Physiopathology of Cancer 338–391 F Homburger & W H Fishman, Eds Hoeber New York, N Y

4 BARRETT, M K 1955 The nature of tumor immunity *In* Origins of Resistance to Toxic Agents 308–333 M G Sevag, R D Reid & O E Reynolds, Eds Academic Press New York, N Y

5 BARRETT, M K , W H HANSEN & B F SPILMAN 1951 The nature of the antigen in induced resistance to tumors Cancer Research **11** 930–936

6 BARRETT, M K & W H HANSEN 1953 Resistance to tumor implantation induced by red cell stromata Cancer Research **13** 269–275

7 WEISS, P 1949 Differential growth *In* The Chemistry and Physiology of Growth . 135–186 A K Parpart, Ed Princeton Univ Press Princeton, N J

8 BILLINGHAM, R E 1956 Investigation of the nature of the antigenic stimulus in transplantation immunity 34th Ann Rept Brit Emp Cancer Campaign 479–485

9 BILLINGHAM, R E , L BRENT & P B MEDAWAR 1956 Quantitative studies on tissue transplantation immunity III Actively acquired tolerance Phil Trans Roy Soc London Ser B **239** 357–414

10 BILLINGHAM, R E , L BRENT & P B MEDAWAR 1956 The antigenic stimulus in transplantation immunity Nature **178** 514–519

11 MERWIN, R M & E L HILL 1954 Fate of vascularized and nonvascularized subcutaneous homografts in mice J Natl Cancer Inst **14** 819–839

12 BREYERE, E J , JR 1957 Several immunogenetic responses of the rat as determined by skin transplantation Dissertation Abstr **17**· 2386

13 SNELL, G D , P SMITH & F GABRIELSON 1953 Analysis of the histocompatibility-2 locus in the mouse J Natl Cancer Inst **14** 457–480

# HOMOLOGOUS WHOLE BLOOD AS AN AGENT FOR ENHANCEMENT OF SKIN GRAFTS IN THE ADULT RABBIT A PRELIMINARY REPORT*

*By* Richard B Stark, Harry Brownlee, and Ronald P Grunwald
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Owen's classic work, showing that a synchorial placental circulation in dizygotic twin freemartin cattle that freely circulates blood cells of the twins produces offspring tolerant of each other's skin transplants, has spurred work upon artificial induction of perinatal tolerance to homografts through the use of cellular injectants [1] The Bar Harbor, Me , group has induced prolonged homograft survival in the adult through the administration of killed cellular elements, achieving what they have termed "enhancement" of homotransplants [2] These important findings suggested the possible beneficial effect of injections of circulating blood elements in the adult

On the debit side of such a theoretical suggestion, however, are the "bloodless" tissues of cornea and cartilage that survive for long periods following homotransplantation Then, also, the common denominator of virtually all tissues and organs that are rejected as homotransplants is the blood cell

Consequently, we set up a pilot experiment to determine what role, if any, homologous circulating blood elements play in the reaction of rejection of homografts of skin We used the ears of rabbits In one ear we injected whole blood subcutaneously Following this injection we transplanted a homograft of full-thickness skin upon the other ear of the same animal We felt that we should elicit accelerated loss of the skin graft in a manner akin to the second-set phenomenon if whole blood cells induce the reaction of rejection of homotransplants If, however, live circulating blood elements produce the phenomenon of enhancement in the adult, we felt that we should elicit prolongation of homograft survival

## *Method*

Transplants of homologous skin were made between adult rabbits of disparate strains (New Zealand Whites and Flemish Giants) In all, 98 rabbits were used in these experiments The rabbit ear serves as a useful recipient site upon the host It is a clean member of the body, rarely touching the floor of the cage and is rarely disturbed by this incurious animal It is surfaced with relatively thin skin and short hair over a thin plate of hyaline cartilage Transplants sutured into place upon the perichondrium of the ear are maintained at normal skin tension by the cartilage, assuring a minimum of technical failures Observations are facilitated, since no dressings are required [3]

Operative transplants were performed using veterinarian pentabarbital sodium (Nembutal) and aseptic precautions Skin grafts were sutured into

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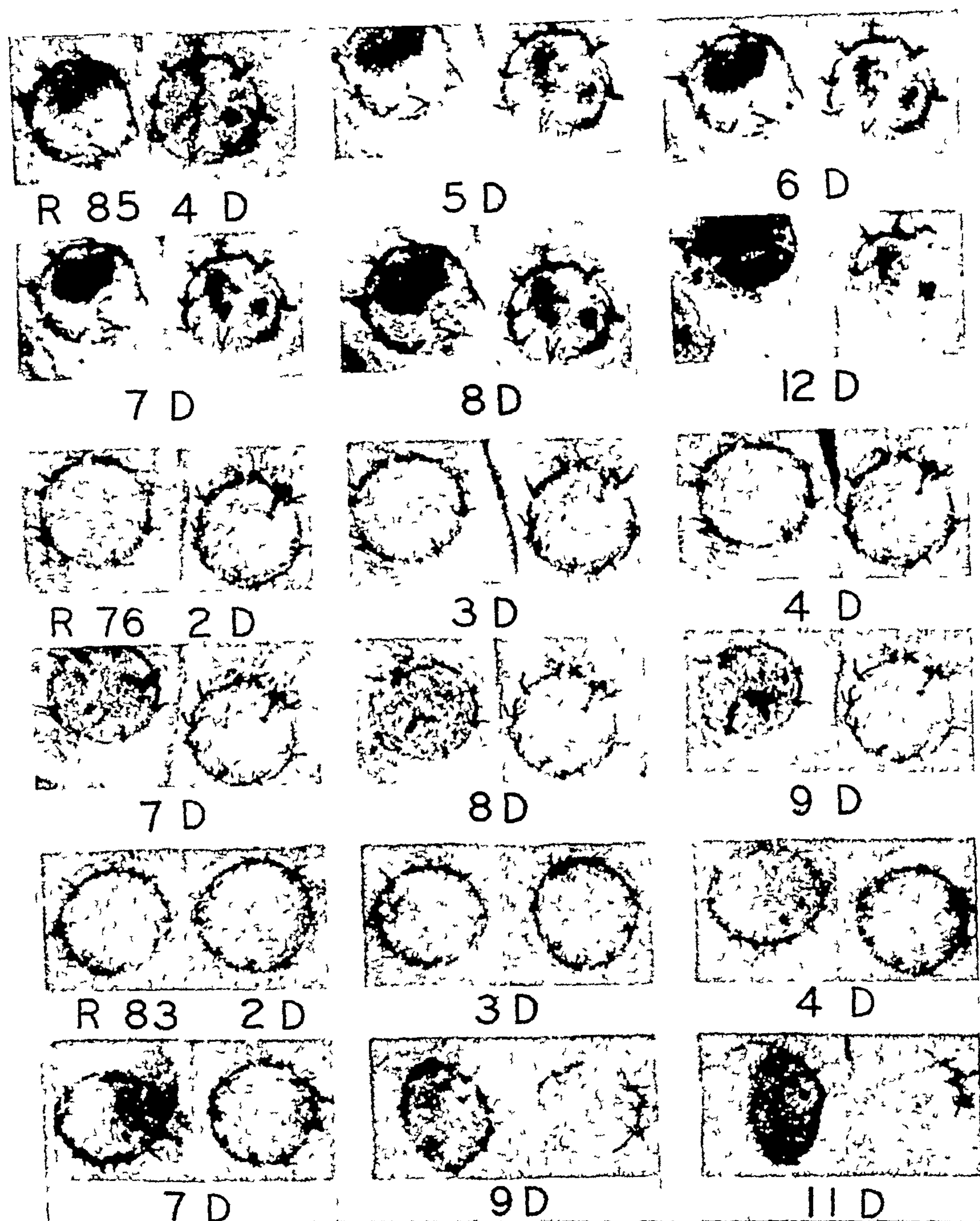



FIGURE 1 Initial studies of first-set homografts and autografts of skin, using the ear of the rabbit both as donor and as recipient site These paired photographs show the macroscopic results of homograft and autograft upon successive periods of observation in 3 rabbits (Read these photographs across ) In rabbits 85, 76, and 83 the homograft in each pair of photographs is on the left, and the autograft is on the right From macroscopic and microscopic studies, over one half of the surface area of the homotransplant showed dry gangrene by the fifth day, at which time necrosis and multinucleated cells were present to the maximum degree

place using eight 3-0 black sutures Earlier studies upon homotransplants reported in the literature utilized skin grafts of small size upon which consecutive biopsies were virtually impossible Therefore, round homografts of skin 2 5 cm in diameter were used

Daily macroscopic observations were made upon the transplants, which were photographed, using color film

The easy availability of the ear and its relative thinness facilitated the taking of consecutive biopsies, which were obtained using a leather punch Such a biopsy produced a tissue sandwich with the transplant upon one side of the cartilage and normal skin on the other to serve as a control All biopsies were read and graded by an impartial pathologist (R G ), who did not have access to the key for identification of specimens

*Results*

Base line studies were made upon autografts and first- and second-set homografts of skin Autografts of skin were uniformly successful First-set homo-

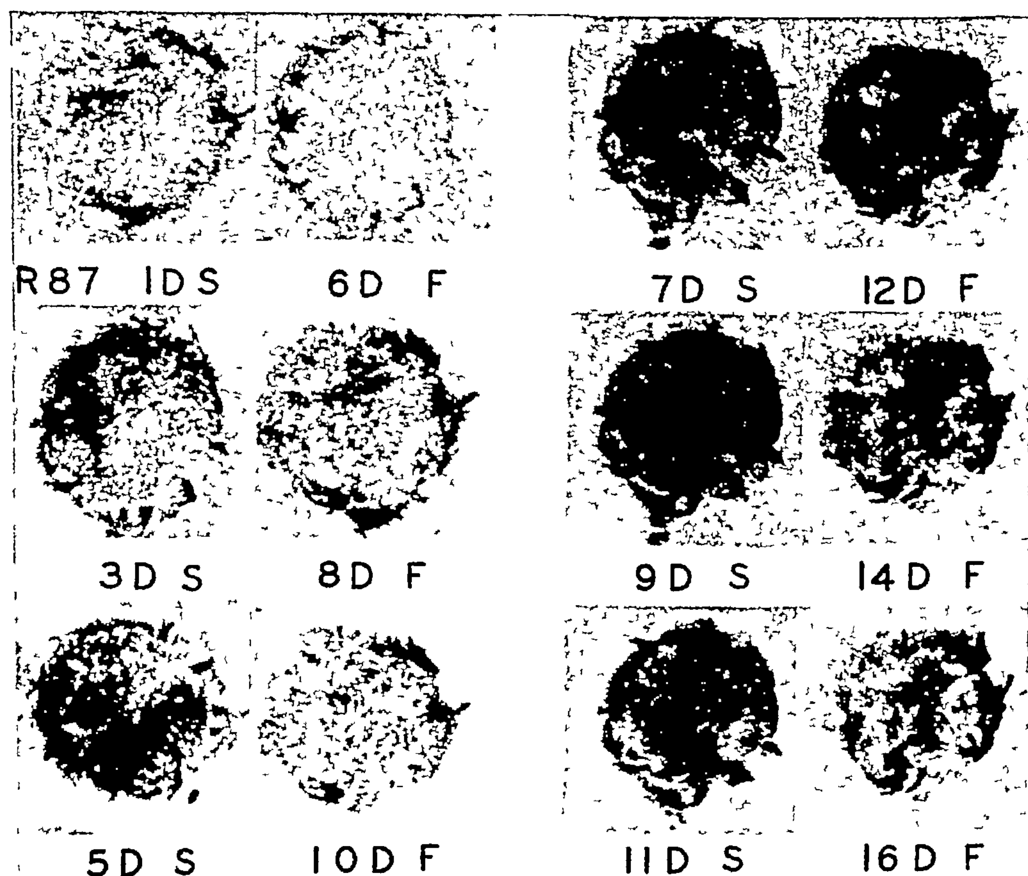



FIGURE 2 The findings from first-set homografts suggested that the reaction of rejection was present in force by the fifth day Consequently, second-set grafts were transplanted at that time Involvement of the entire transplant with dry gangrene occurred by the second day, and necrosis was evident by biopsy at that time Mononucleated cells did not appear at that time, however (Read these photographs downward First-set homografts are marked "F" and second-set grafts are marked "S" The number of days post-transplantation is also marked )

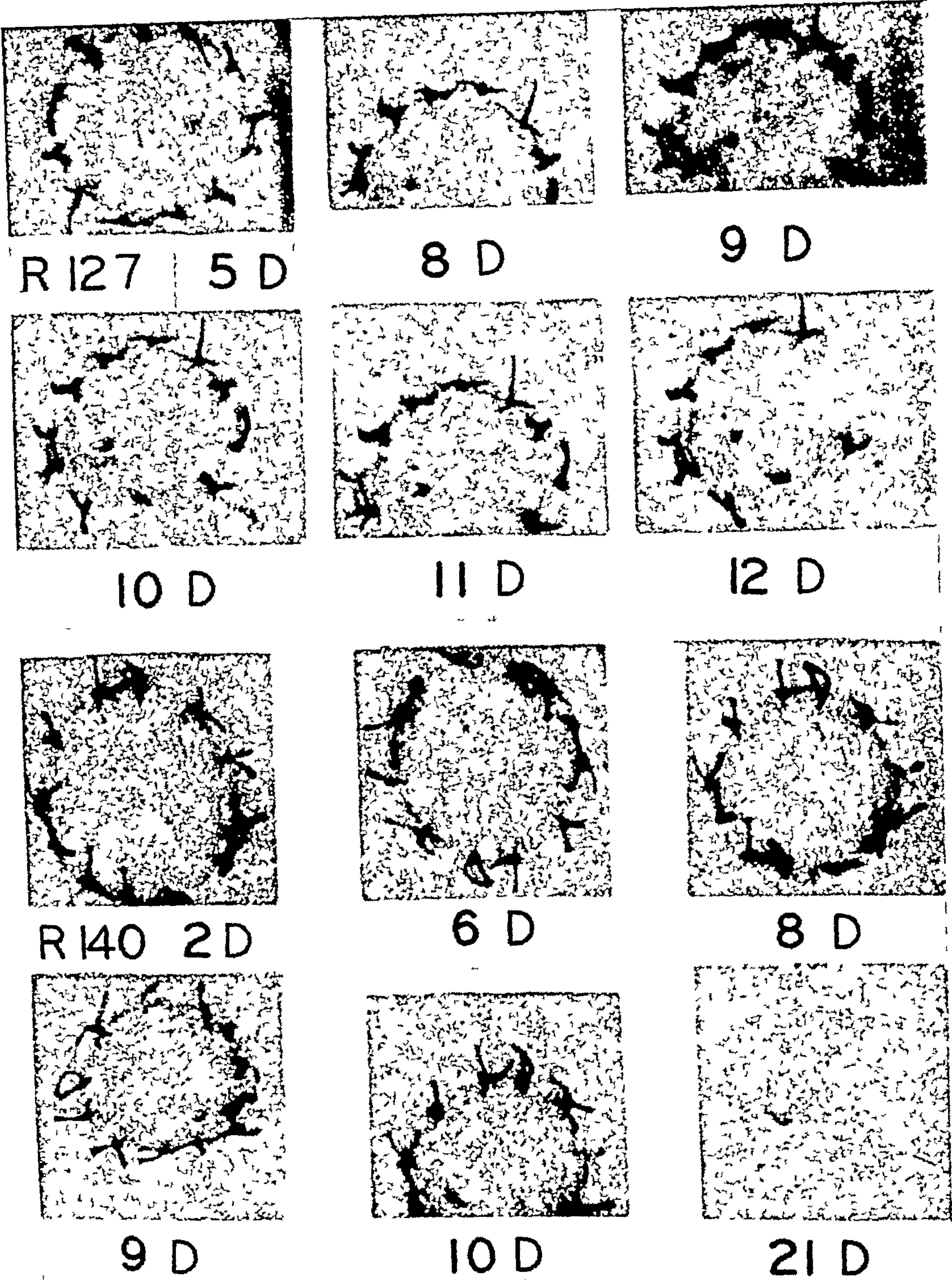



FIGURE 3 To assay its role in the homoplasty reaction, whole blood from a donor of a disparate strain was injected subcutaneously into rabbits Five days later a skin homograft was transplanted from the blood donor to the same recipient Instead of accelerating the loss of the skin homotransplant, whole blood injection produced a prolongation of homograft survival and an amelioration of the reaction of rejection (Read these photographs of rabbits 127 and 140 across )

grafts of skin showed macroscopic necrosis of 50 per cent or more of their surface area by the fifth day, at which time necrosis and the presence of mononucleated cells were at their maximum, as evidenced by biopsy (FIGURE 1) We interpreted this as implying that the reaction of rejection was in force. Consequently, we chose this time (5 days) to transplant second-set homografts of skin upon the opposite ear of the same host.

Macroscopically, second-set homografts became necrotic earlier than the first-set grafts (FIGURE 2) Necrosis comparable to that exhibited by the first-set grafts upon the fifth day was exhibited by the second-set grafts upon the second day There was little evidence of mononucleated cells, however, in the second-set biopsies This is interesting in the light of the current hypothesis that mononucleated cells mediate the homoplastic reaction of rejection

Next, homologous whole blood, obtained by intracardiac puncture from the future skin donor, was injected subcutaneously (1 cc) into one ear of the recipient (FIGURE 3) Upon the fifth day following injection, when the reaction of rejection (if present at all) should have been present in force, we transplanted a homograft of skin from the blood donor to the opposite ear of the same recipient

What we observed in all rabbits so treated was not a second-set phenomenon with rapid slough of the skin homograft transplanted just after the blood injection, but rather a prolongation of the life of the homograft of skin (as evidenced by macroscopic and microscopic observations) and an amelioration of the reaction of its ultimate rejection[4] In all rabbits treated thus the life of the homotransplant of skin was prolonged appreciably more than 100 per cent. Our numbers are yet too small to be statistically significant, but we have not seen prolongation of the life of the homograft of lesser magnitude than this

### *Discussion*

It is pointless to speculate upon the *modus operandi* of this phenomenon of enhancement of skin grafts in the adult rabbit, using live homologous whole blood cells, until additional work has been done We can say that, by using this easily applied modality, the life of homotransplants of skin appears to be prolonged in the adult rabbit in spite of the use of a relatively small amount of enhancing substance

### *Acknowledgment*

We are indebted to Margy DeForest and to Rudolph Garet for their not inconsiderable aid in this project

### *References*

1 OWEN, R D 1945 Immunogenetic consequences of vascular anastomosis between bovine twins Science, **102** 400
2 SNELL, G D, A. M CLOUDMAN, E FAILOR, & P DOUGLASS 1946 Inhibition and stimulation of tumor homoiotransplants by prior injections of lyophilized tumor tissue J Natl Cancer Inst 6 303
3 STARK, R B 1957 The rabbit ear as an experimental tool Plastic Reconstr Surg 19: 28
4 STARK, R B Current concepts of the homoplastic enigma Bull N Y Acad Med In press

# IV. Graft-Versus-Host Reaction and Acquired Tolerance in Tissue Homotransplantation

## INTRODUCTION PROBLEMS IN TRANSPLANTATION IMMUNITY

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This paper deals with three aspects of transplantation immunity The first is graft acceptance, about which we know little and about which we speculate rarely The second is acquired immunity to grafts, a subject about which we have gained a reasonably good body of knowledge The third is acquired tolerance, concerning which we have learned much and speculate still more

*Graft acceptance* Primary acceptance of grafts has not been of much interest to immunologists One hears at times that immune factors have but little influence on the initial fate of a transplant, and that it is not until the host has had time to develop an immune response against the graft that immune forces become decisive However, previous contacts of the host with antigens similar to those of the graft influence the reception of the graft by the host, undoubtedly these contacts frequently prevent even initial establishment of the graft The fact that some bodily sites, such as the brain and the eye, are more hospitable to grafts than others, at least initially so, could be ascribed to the fact that these sites are relatively sheltered from immune processes that have run their course prior to the grafting procedure [1]

Whatever the influences of such earlier immune events may be, they are probably only of secondary importance The initial failure of a graft or, rather, the inability of a graft to establish itself, occurs frequently when the host does not object to the graft from an immunological point of view The transfer of an entire brain or an entire heart from a mouse of one inbred strain to the peritoneal cavity of another mouse of the same inbred strain will not be successful However, one need not quote extreme examples Transfers of tumors, particularly lymphomas, within an inbred strain of mice are frequently unsuccessful, and plastic surgeons have their share of experience with failures of skin autografts This sort of initial failure lies in the field of "plumbing," and often can be prevented by establishing better channels for oxygen supply and drainage One should keep in mind that improved plumbing made possible the successful transfer of kidneys, and that failures because of insufficient plumbing are more frequently encountered with a more intricate vasculature of a graft and with cells that are not resistant to temporary anoxia Theoretically, every cell, every organ, is transplantable, provided host and graft are immunologically compatible It is irksome to hear investigators use such terms as transplantable and nontransplantable tumors It would be just as proper to speak of transplantable and nontransplantable skin

Problems of graft acceptance are related to the phenomena of regeneration and growth It is therefore unlikely that the questions surrounding initial acceptance of a graft can be answered until we known more about regeneration and growth, about which there are many unknowns and variables It is un-

likely that we can solve the problems of graft acceptance by using the techniques of homografting or heterografting, procedures in which the number of unknowns and variables is considerably higher. However, problems of graft acceptance are of importance primarily in heterografting, since successful heterografts, except in unusual circumstances, do not survive the phase of graft acceptance and, as a rule, are swept away by the powerful immune response to the antigens of the foreign species. Therefore, experiments in heterografting intended to contribute to fundamental understanding of biological events are directed toward aims that may well be beyond reach.

*Acquired immunity.* Circulating antibodies to grafts, *in vivo* or *in vitro*, are readily demonstrable in heterografting. The problem becomes more difficult in homografting, particularly homografting of normal tissues such as skin. Hemagglutinins and hemolysins are frequently encountered following the grafting of tumors of foreign strains, but it is questionable whether these hemagglutinins play an active role in the destruction of tumor grafts. In the future, improved methods may reveal effective circulating antibodies, but it is possible that there are none and that the destruction of skin homografts is brought about by antibodies attached to the surface of intact host cells. The experiments with grafts enclosed in millipore filters, chambers whose pores are adequate to permit the passage of protein molecules but too small to permit passage of host cells, are of particular interest in this regard. In interpreting these experiments one should consider, however, that while too small a pore may prevent passage of too large a particle, it also may interfere with the passage of particles considerably smaller than its diameter. A screen door not only prevents the entrance of flies and mosquitoes, but also interferes with the free flow of air.

The studies in our laboratory have been concerned chiefly with the nature of graft antigens. Grafts of male mouse skin are rejected by females of the same strain. It is generally assumed that this rejection is due to an antigen determined by a histocompatibility gene located on the Y chromosome. We have hesitated to accept this explanation without reservations, although it is compatible with our observations. Our hesitation is based on studies in *Drosophila* by Fox.[2] Sex determination in *Drosophila* appears more complicated than in mammals, although *Drosophila* carries but four pairs of chromosomes. While many females have the standard XX chromosomes and males the XY chromosomes, other females carry also the Y chromosome (XXY), and some males lack it (XO). If sex determination is so complicated in a species having only four pairs of chromosomes, it is likely to be even more complicated when the number is much higher. The fact that there are no relevant observations to suggest this possibility in mammals may be due merely to the greater complexity of the chromosomal picture.

Our more recent studies have been concerned primarily with the specificity of the male antigen and possible additional sex-linked antigens. The problem of strain specificity presented itself to us when we noted that $F_1$ hybrid males between two inbred strains accepted each other's skin grafts even when they were of reciprocal parentage. This should not have occurred if the antigens determined by the Y-linked genes differed from each other, provided both

strains carried such a gene Second, we noted that a graft from a male of one pure parent strain of an $F_1$ hybrid female was able to induce in this female a second-set response to a graft from a male of the other pure parent strain Third, we observed the growth of a tumor (Tumor 58) that we had induced with benzopyrene in a C57BL male The tumor grew significantly faster in untreated controls than in pretreated animals The pretreatment consisted of injecting normal tissues from the non-C57BL parent strain into female $F_1$ hybrids from crosses of C57BL with C3H, A, Street, and BALB/c strains All of these observations gave additional support to our assumption not only that the Y-linked gene was present in all strains examined by us, but also that the antigen determined by it was not strain specific

It occurred to us that the X chromosome, which is much larger than the Y chromosome, also might carry a histocompatibility gene If it did, as explained by Haldane,[3] male $F_1$ hybrids should reject male or female skin grafts of the paternal parent strain (whose X chromosome this hybrid lacked), while grafts of the maternal parent strain should be accepted Clearly, this course of events could happen only if the hypothetical X-linked gene were strain specific and if it would elicit an immune response sufficiently severe to cause rejection One should expect also that $F_1$ hybrid males whose paternal strain is C57BL and the maternal strain non-C57BL would show retardation of growth of Tumor 58 as compared to growth of the same tumor in its reciprocal Peculiarly, the opposite happened, the tumor grew faster in $F_1$ hybrid males whose paternal strain was C57BL This observation suggests that additional male antigens are present in the C57BL strain, but absent in other strains an observation that we are attempting to confirm at present

*Acquired tolerance* The concept of tolerance due to chimerism acquired during phases of immunological incompetence is disturbing and not easy to accept In our own limited experience with the grafting of normal tissues, biological relationships usually are not that simple and clear cut There is also the possibility that one listens more keenly to positive than to negative results, although the latter eventually may provide significant clues

A common observation difficult to understand is that tolerance seems readily achievable with certain donor-host combinations, but only with difficulty in others Also, some investigators have had bad luck with C57BL cells, while others report complete success It is not clear why this should be the case

In attempting to create tolerance we are faced with tasks in newborn mice different from those in irradiated adult mice In the former we are striving only for tolerance, in the latter we try to supply protection against irradiation as well as to induce tolerance The number of cells needed to induce tolerance in the newborn, to provide protection against irradiation, and to induce tolerance in the protected mice are of the same general order of magnitude between 3 million and 30 million cells Still, this similarity may be chance Another observation is puzzling insuring survival after irradiation requires larger numbers of foreign cells than cells of the animal's own strain While this may seem acceptable at first glance, it is disturbing on second thought Either the host accepts the foreign cells as if they were its own because its own defenses are inadequate, or these defenses are adequate and the host rejects these cells,

the only benefit the host could then derive from such rejected, disintegrating cells would be some temporary humoral principle. However, some investigators believe that the foreign cells survive and persist for a long time.

A recently reported observation by Main and Prehn[4] is also of considerable interest. While it is logical that an irradiated mouse of one strain can be protected by a sufficient number of competent cells of its own or a different strain, these investigators noted that protection also can be achieved by a mixture of cells of both strains. Both types of cells survive, and the animal is tolerant of skin grafts from either strain. Why is it that the injected, undamaged, competent cells of both strains do not dispose of each other?

There is much evidence that procedures producing chimerism are often followed by symptoms of "runt" disease caused, as we believe, by a reaction of injected cells against the host. Various investigators disagree as to the findings. According to some, the spleen of a runt is too small, according to others, it is too large. Again, the type of donor-host combination seems to be of importance. What is it, then, that causes tolerance, and what is it that causes runt disease? Is it only a quantitative difference, and is runt disease tolerance in the extreme? Is tolerance acquired because of the presence of antigen-carrying and antigen-producing cells, and does runt disease develop only when these cells also are antibody producers? If this were true, it would create a dilemma in postirradiation tolerance where antibody-producing cells are very much needed to ward off the irradiation damage, but where any additional contribution in antibody production would be most unwelcome. The situation would be simpler in the injection of newborns where antigen production alone would be needed for tolerance and where one might advantageously select cells, such as epidermal or renal tubular cells, that carry antigen, but are not known to be strong antibody producers. A liver cell suspension probably would be less suitable. Several investigators have turned to fetal or embryonal cells that carry antigen, but are deficient in antibody production. What will these immature cells do when they mature in the tolerant host? Is that the time at which they would produce runt disease?

When cells of an inbred strain are injected into an adult $F_1$ hybrid we are dealing with a situation of chimerism for the production of which neither irradiation nor the newborn state are needed. While the hybrid accepts the parental cells without protest, the parental cells are free to develop the same homograft response that a pure parent develops against an $F_1$ skin or tumor graft. It should be a most favorable situation for the development of runt disease, but how do these adult $F_1$ mice react upon receiving large intravenous doses of spleen cells of a pure parent strain? In our experience they grow and gain weight, and there is no evidence of runt disease. It could be argued that any antibody produced by the tolerated donor cells is absorbed by the host tissues, but this argument undermines the explanation of runt disease. What happens to the injected spleen cells? Do they not survive? What would be a good method to test survival of parental cells in $F_1$ hybrids? We have considered injecting the spleen cells of a suspected $F_1$ hybrid chimera $\left(\frac{AB}{B}\right)$ into irradiated mice of one of the parent strains (A) that have been sensitized against

cells of the other parent strain (B) prior to irradiation These mice should dispose quickly of the hybrid cells (AB), but would not attack the tolerated chimeric cells (A) Consequently, a protective effect would result if a sufficient number of tolerated cells were present in the spleen cell suspension However, this approach is almost certain to fail because of the small number of tolerated parent cells that, at best, would be present An approach using the antigenic instead of the antibody-producing capacities of tolerated cells would have a greater chance of success

These are some of the questions that come to mind on contemplating acquired tolerance Few questions have captivated the minds of investigators closely and even remotely concerned with tissue transplantation as much as this one has While acquired tolerance seems a significant break through the solid wall of the homograft response, it is doubtful that it brings us actually closer to a solution of the homograft problem Probably it will bear at least as much fruit in other fields of endeavor

*References*


1 RAMBO, O N , JR , R FUSON, M HATTORI & E J EICHWALD 1954 Immune phenomena elicited by transplanted tumors I The participation of the eye and the brain Cancer Research **14:** 169

2 FOX, A S 1956 The detection of antigenic differences attributable to the Y chromosome Transplantation Bull **3:** 131

3 HALDANE, J B S 1956 The detection of antigens with an abnormal genetic determination J Genetics **54:** 54

4 MAIN, J M & R T PREHN 1957 Fate of skin homografts in x-irradiated mice treated with homologous marrow J Natl Cancer Inst **19** 1053

# STUDIES ON THE REACTION OF INJECTED HOMOLOGOUS LYMPHOID TISSUE CELLS AGAINST THE HOST*

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With some combinations of inbred strains of mice, such as CBA → A and vice versa, it has been found that the intravenous injection of newborn animals with a dosage of 4 to 10 million living homologous adult spleen cells will induce a high degree of tolerance of subsequent skin homografts from the donor strain in a great proportion of the subjects [1, 2] Inoculation of the newborn animals by the intraperitoneal route is less effective in conferring tolerance, and the subcutaneous route is completely ineffective, probably because cells introduced by this route do not reach the developing immunologically competent tissues of the host soon enough At birth the mouse, unlike the rat,[3] is almost at the end of the tolerance-responsive phase of its life

With this method of inducing tolerance in mice, a completely unexpected complication was encountered in certain strain combinations, for example, C57 → A and AU → A, in which all injected animals died within 2 or 3 weeks after an initial period of normal growth and apparent well-being The first indication of disease was cessation of growth, usually accompanied by diarrhea, death followed within a few days Uninjected litter mates, it may be emphasized, remained perfectly healthy and unaffected by the disease that was killing their injected brothers and sisters

With other strain combinations, although most of the mice appeared to be suffering from this disease, its severity was variable a few of the victims died soon after injection following an "acute" attack, others suffered from what appeared to be a chronic, sublethal attack that retarded their growth so severely that it was by no means unusual to find animals with body weights of no more than 5 to 7 gm at 50 to 70 days of age (see FIGURE 1)

Fortunately, from the point of view of obtaining tolerant animals, a variable proportion of the mice, depending upon the particular strain combination, either failed to show any clinical symptoms of this disease or recovered from what appeared to be a very mild attack As TABLE 1 shows, with nearly all strain combinations tested a fairly high proportion of the surviving mice were found to be tolerant when challenged with a skin homograft from the donor strain of the neonatal spleen cell inoculum Unfortunately, because of their small size and feeble condition, only a small proportion of the runts could be test grafted, but all those that survived this operation proved to be fully tolerant for as long as they lived

The subject matter of this paper is an investigation of the etiology of this peculiar disease, referred to descriptively as "runt disease," which is associated with the inoculation of newborn mice with adult homologous spleen cells Some of the work described in this paper was carried out in collaboration with Leslie Brent of University College, London, England

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FIGURE 1 Three 52-day-old A-strain mice from a litter that was injected at birth with 5 million adult C3H spleen cells Two of these mice are chronic runts, weighing only 6 and 7 gm, respectively Note the emaciated appearance and scruffy condition of the fur of the smaller animal The larger, apparently unaffected animal was fully tolerant of a subsequent C3H skin homograft, the other 2 animals were too small to be grafted

TABLE 1

ATTEMPTS TO CONFER TOLERANCE IN NEWBORN MICE OF DIFFERENT STRAINS BY THE INTRAVENOUS INJECTION OF LIVING HOMOLOGOUS ADULT SPLEEN CELLS

| Strain combination | Mortality, per cent | Number grafted | Tolerant, per cent | Highly tolerant,* per cent |
|---|---|---|---|---|
| CBA → A | 10 | 53 | 96 | 80 |
| A → CBA | 21 | 23 | 56 | 39 |
| C3H → A | 36 | 15 | 100 | 87 |
| A → C3H | 67 | 10 | 70 | 43 |
| C3H → CBA | 0 | 5 | 100 | 100 |
| CBA → C3H | 0 | 12 | 100 | 100 |
| A → C57 | 6 | 17 | 6 | 0 |
| C57 → A | 100 | — | — | — |
| A → AU | 47 | 10 | 60 | 50 |
| AU → A | 100 | — | — | — |
| CBA → AU | 22 | 20 | 25 | 5 |
| C57 → AU | 89 | 1 | 100 | 100 |
| C57 → CBA | 100 | 0 | — | — |

All mice were injected within 24 hours after birth
Spleen cell dosage per mouse 4 to 10 million
Under mortality is included the percentage of the injected mice whose deaths were attributable to acute or chronic runt disease This figure does not include mice that died as an immediate consequence of the neonatal inoculation or as a result of maternal neglect
* Highly tolerant mice are those that accepted their test grafts for at least 50 days

### *Abnormalities of the Lymphoid Tissues of Tolerant Mice*

Careful dissection of healthy, clinically normal-looking mice which, as a consequence of their neonatal exposure to homologous spleen cells, were fully tolerant to skin from the donor strain, almost invariably revealed evidence of abnormalities or involution of their lymphoid tissues[2] This was reflected in gross reductions in size, or even the apparent absence of some of their nodes, or in the exceedingly flabby and unhealthy looking condition of some of these organs Even Peyer's patches were not infrequently involved The spleens of these animals were usually normal in appearance

In incompletely tolerant mice examined after the breakdown of their test grafts, abnormalities of this type were seen in less than 50 per cent of them and were very trivial when present Mice that gave no indications of being tolerant had perfectly normal nodes

It was in the runts that this involutionary trend in the lymphoid tissues of injected mice reached its climax In such animals it was usually practically impossible to identify any of their nodes Also, their spleens, though usually of apparently normal size, were discolored, fibrotic, and grossly deficient in Malpighian corpuscles Besides retardation of growth, involution of lymphoid tissue was another constant and diagnostic symptom of runt disease, for fully tolerant mice that, as already stated, had never shown any overt symptoms usually displayed some abnormalities with respect to their nodes

### *Two Possible Causes of Runt Disease*

There are two obvious but completely different hypotheses that could account for the facts thus far presented[2] According to the first of these, runt disease is the outcome of a concurrent transmission of pathogens, along with the homologous spleen cells, from strains of mice to which they have become adapted into members of potentially susceptible strains at a highly vulnerable age This hypothesis has many serious shortcomings[4] It fails to account for the fact that many fully tolerant mice remained normal, healthy indiviuals, yet showed one important symptom of the disease, namely, involution of their nodes It is not supported by the experimental findings with potentially lethal strain combinations, such as AU or C57 → A, in that (1) inoculation of newborn mice with plasma is harmless, (2) only a small proportion of animals died following their inoculation with 0 05 ml of C57 whole blood, (3) although intraperitoneal inoculation of C57 spleen cells was just as lethal as inoculation by the intravenous route, subcutaneous inoculations produced no adverse effects, and (4) inoculation of cell suspensions prepared from bone marrow did not kill the recipients Other direct experimental evidence that tells very strongly against this "infection" hypothesis has been presented by Brent[5]

The second hypothesis ascribes runt disease to an immunological reaction, that is, a homograft reaction, on the part of the inoculated homologous spleen cells against the "transplantation" antigens of their "foreign" host The prima facie case for this hypothesis is a strong one Spleen cells are known to be immunologically competent, and the tolerance that they confer enables them to survive for considerable periods By employing a test, the principle of which

was devised by Mitchison,[6] it has been established that the cells inoculated into newborn mice, or their mitotic descendants, persist and are present in the nodes, spleens, thymuses, kidneys, and bone marrow, and also in the leukocyte population of the circulating blood of fully tolerant adult mice, these animals are therefore cellular chimeras. Presumably the inoculated spleen cells colonize anatomically appropriate environments in their developing hosts, such as the nodes and various aggregations of lymphoid tissue in other organs. The organs overtly affected in runt disease are, therefore, those that the inoculated cells are known to colonize. The observed differences in the severity of the disease with the different donor/recipient strain combinations tested are explicable on a simple immunogenetic basis: provided that the inoculated cells secure for themselves a protective cloak of tolerance, the severity of their reaction against the host should be determined by the antigenic disparity between their host and themselves. The evidence thus far obtained is in accord with this interpretation. The incidence of runt disease is greater with the more remotely related strains, with the very closely related strain-combination C3H → CBA and vice versa all the inoculated mice became highly tolerant, and none manifested the slightest detectable abnormality of its lymphoid tissues. Finally, our finding with the potentially lethal combination C57 → A that the inoculation of 4-day-old mice with spleen cells is completely harmless is just what one would expect if runt disease depended upon the prolonged survival and immunological reactivity of the cells, because the 4-day-old mouse is already too old to be tolerance responsive.[2]

The experiments described below all yield evidence that is either consistent with the graft-versus-host hypothesis or provide experimental confirmation of predictions based upon it.

*Inoculation of Newborn Mice with Spleen Cells from Hybrid Donors*

According to the graft-versus-host hypothesis, whereas the injection of newborn A-strain mice with AU or C57 spleen cells invariably causes acute runt disease, the inoculation of these baby mice with hybrid spleen cells from C57/A or AU/A donors should be completely harmless, since both types of cells should be genetically incapable of reacting against antigens common to themselves and to their hosts. At the same time, these hybrid cells should confront their hosts with all the antigens of the C57 or AU strains. This prediction has been fully sustained experimentally, the hybrid cells were completely innocuous to their A-strain hosts and conferred tolerance upon many of them.[4]

These experiments are of considerable importance, since they make it perfectly clear that, although runt disease depends upon tolerance, tolerance in no way depends upon the involution of lymphoid tissue associated with runt disease. Other evidence bearing upon this important point has been summarized elsewhere.[4, 5]

*Inoculation of Newborn A-Strain Mice with Other Types of C57 Cells*

In attempts to confer tolerance without the intervention of runt disease, the members of newborn A-strain litters have been injected with cells prepared

from adult C57 or AU donor tissues other than the spleen. It has been found that leukocyte concentrates prepared from the buffy coat of whole blood and containing as few as one million of these cells invariably caused acute runt disease. This provides strong proof that at least one cellular ingredient of the leukocyte population of adult mammalian blood is immunologically competent and capable of proliferation—a finding that parallels Simonsen's observation in chickens[7] and may be of significance in interpreting the etiology of certain types of hemolytic disease of the newborn in man, since it hints at the possible consequences of maternal leukocytes crossing the placental barrier and gaining access to the fetus. The fact that inocula comprising about 0.05 ml. of whole blood were rarely harmful is not surprising, because the number of leukocytes present in this volume is insufficient to represent a tolerance-conferring stimulus.

Excluding the spleen, the thymus represents the only convenient alternative source of large numbers of dissociable viable cells. When newborn A-strain mice were inoculated with 5 to 10 million thymocytes from adult C57 donors, the majority died of acute runt disease and none of the survivors showed the slightest degree of tolerance when subsequently grafted with C57 skin. This finding lends strong support to the view that the thymus is capable of mediating immunological reactions, though the variability of the results has yet to be accounted for.

When suspensions containing about 10 million dissociated bone marrow cells from adult C57 donors were administered to baby A-strain mice, all of them survived and grew up normally. When test grafted, the majority were found to be highly tolerant. However, dissection of such animals revealed that bone marrow cells were by no means as harmless as the outward appearance of these mice had suggested. There was evidence of very severe involution and abnormalities of their nodes, though they were not quite as badly afflicted as the chronic runts. Our tentative interpretation of the finding that C57 marrow cells are relatively less harmful than spleen cells from this donor strain is that the marrow simply contains a smaller proportion of immunologically competent cells than the spleen. It seems likely that, had we employed higher dosages of marrow cells, the hosts would have succumbed to acute runt disease.

*Discussion*

The possibility that homografts might be capable of reacting against their hosts was first suggested independently by Dempster[8] and Simonsen[9] on the basis of histological studies on homotransplanted kidneys. Formal, though indirect, experimental proof of this hypothesis was furnished by the subsequent demonstration that breakdown of a skin homograft on a tolerant mouse took place if normal lymphoid tissue cells from another mouse of its own strain were implanted into the tolerant mouse.[10]

The findings and conclusions drawn from the present study are in complete accord with the results of Simonsen's[7] recent analysis of the etiology of the profound pathological changes in the spleen and other organs of young chicks and of mice following their injection, before or soon after hatching or soon after birth, with homologous adult spleen or buffy coat cells. The facts that there

exists a complete theoretical basis for the graft-versus-host hypothesis of the origin of the various pathological changes in young animals injected with adult, immunologically competent lymphoid tissue cells, that every experimental fact may be drawn as an inference from it, and that it successfully emerges from a whole series of critical tests, constitute such a strong case for its validity that further discussion of the "infection" hypothesis would be futile

In theory and in practice the induction of tolerance in young animals is not the only condition for the revelation of graft-versus-host reactions, although in all probability the newborn mouse or young chick probably afford the most susceptible test subjects

Evidence has been obtained that cellular homografts of immunologically competent tissues from a parental strain can harm and may sometimes kill their hosts if introduced into fairly young $F_1$ hybrid mice, a situation that takes advantage of a genetically "in-built" tolerance [4, 11]

When mice are protected with homologous, or even heterologous, adult spleen or bone marrow cells against the otherwise lethal effects of high doses of X irradiation, conditions requisite for the revelation of graft-versus-host reactions are also realized The foreign cells quite definitely persist in these animals, which are incapable of rejecting them,[6, 12] indeed, their protective effect depends upon their ability to rehabilitate the depleted hematopoietic and lymphoid tissues of the host The very fact that the protection afforded by homologous, as opposed to isologous, cells frequently is followed sooner or later by deaths attributed to "secondary disease" or to "homologous disease," the symptoms of which bear a close resemblance to those of "runt" disease,[13, 14] suggested to Trentin[15] and others that these delayed deaths were due to immunological reactivity on the part of the injected cells Formal proof of this concept has now been presented [16, 17]

*References*


1 BILLINGHAM, R E & L BRENT 1956 Acquired tolerance of foreign cells in newborn animals Proc Roy Soc London **B146** 78–90
2 BILLINGHAM, R E & L BRENT 1957 A simple method for inducing tolerance of skin homografts in mice Transplantation Bull **4:** 67–71
3 WOODRUFF, M F A & L O SIMPSON 1955 Induction of tolerance to skin homografts in rats by injection of cells from the prospective donor soon after birth Brit J Exptl Pathol **36:** 494–499
4 BILLINGHAM, R E 1958 The reaction of injected homologous lymphoid tissue cells against the host Coll Intern Biol Homogreffes In press
5 BRENT, L 1958 The reaction of splenic grafts against tolerant hosts Conference on Biological Basis of Tissue Transplantation Prague In press
6 MITCHISON, N A 1956 The colonisation of irradiated tissue by transplanted spleen cells Brit J Exptl Pathol **37.** 239–247
7 SIMONSEN, M 1957 The impact on the developing embryo and newborn animal of adult homologous cells Acta Pathol Microbiol Scand **40** 480–500
8 DEMPSTER, W J 1953 Kidney homotransplantation Brit J Surg **40.** 447–465
9 SIMONSEN, M 1953 Biological incompatibility in kidney transplantation in dogs Acta Pathol Microbiol Scand **32·** 1–35
10 BILLINGHAM, R E, L BRENT, & P B MEDAWAR 1955 Acquired tolerance of skin homografts Ann N Y Acad Sci **59** (3) 409–415
11 COLE, L J, & M E ELLIS 1958 Science In press
12 FORD, C E, J L HAMERTON, D W H BARNES & J F LOUTIT 1956 Cytological identification of radiation-chimaeras Nature **177.** 452–454
13 CONGDON, C C & I URSO 1957 Homologous bone marrow in the treatment of radiation injury in mice Am J Pathol **33.** 749–767

from adult C57 or AU donor tissues other than the spleen It has been found that leukocyte concentrates prepared from the buffy coat of whole blood and containing as few as one million of these cells invariably caused acute runt disease This provides strong proof that at least one cellular ingredient of the leukocyte population of adult mammalian blood is immunologically competent and capable of proliferation—a finding that parallels Simonsen's observation in chickens[7] and may be of significance in interpreting the etiology of certain types of hemolytic disease of the newborn in man, since it hints at the possible consequences of maternal leukocytes crossing the placental barrier and gaining access to the fetus The fact that inocula comprising about 0 05 ml of whole blood were rarely harmful is not surprising, because the number of leukocytes present in this volume is insufficient to represent a tolerance-conferring stimulus

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# EFFECT OF PREINJECTION OF HOMOLOGOUS LEUKOCYTES ON HOMOTRANSFER OF LYMPH NODE CELLS IN RABBITS*

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## *Introduction*

This report presents recent data from a series of investigations that originated as an immunological study involving cell transplantation and has developed aspects of a cell transplantation study involving immunology

Earlier studies had shown that following the transfer to fresh recipient rabbits of popliteal lymph node cells of donor rabbits previously injected in the foot pad with *Shigella paradysenteriae*, agglutinins appeared in the sera of the recipients[1] Subsequently it was found that following the transfer of donor lymph node cells incubated *in vitro* with a filtrate of trypsin-treated suspensions of *Shigella* (ST filtrate) agglutinins also appeared in the sera of the recipients[2] The donors and recipients were chosen at random from a heterogeneous population of rabbits

This sequence of events was markedly affected when leukocytes pooled from the prospective donors were injected intradermally into the prospective recipients prior to cell transfer If leukocytes were preinjected in suitable numbers at an appropriate interval prior to the lymph node cell transfer, agglutinins to *Shigella* failed to appear thereafter in the sera of the recipients[3] An exploration of some aspects of the problem is reported below

## *Variations in the Interval Between Preinjection of Donor Leukocytes and Cell Transfer*

When lymph node cells incubated *in vitro* with ST filtrate were transferred to irradiated recipient rabbits, agglutinins appeared in the sera of these recipients on the fourth day after transfer and reached a maximum titer on the sixth to eighth day If $10^7$ pooled donor leukocytes were injected intradermally 1 day prior to cell transfer, agglutinins appeared as usual after transfer When leukocytes were injected 2 days before transfer a number of the recipients developed agglutinins as usual, and a number developed agglutinins to markedly lower levels, the geometric mean of the peak titers of the entire group being distinctly lower than the mean for the control groups (not preinjected) With increasing intervals between the injection of donor leukocytes and transfer of lymph node cells the subsequent agglutinin titers in sera of recipients decreased until, at an interval of 6 days, almost none of the recipients showed serum agglutinins The individual peak agglutinin titers and the means for each group are shown in FIGURE 1 As the interval between preinjection and cell transfer was increased to 20 days the same results were obtained At an interval of 35 days it was observed that the mean titer of the recipients began to rise, and

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14 Barnes, D W H & J F Loutit 1956 Immunological and histological response following spleen treatment in irradiated mice *In* Progress in Radiobiology J S Mitchell, B E Holmes & C L Smith, Eds . 291–296 Oliver and Boyd Edinburgh, Scotland
15 Trentin, J J 1956 Mortality and skin transplantability in X-irradiated mice receiving isologous, homologous or heterologous bone marrow Proc Soc Exptl Biol Med **92**· 688–693
16 Uphoff, D 1958 Preclusion of secondary phase of irradiation syndrome by inoculation of fetal haematopoietic tissue following lethal total-body X-irradiation J Natl Cancer Inst **20** 625–632
17 Trentin, J J 1957 Induced tolerance and "homologous disease" in X-irradiated mice protected with homologous bone marrow Proc Soc Exptl Biol Med **96** 139–144

*Discussion of the Paper*

J W Ferrebee (*The Mary Imogene Bassett Hospital, Cooperstown, N Y, and the College of Physicians and Surgeons, Columbia University, New York, N Y*) It seems clear from careful presentations such as that of Billingham that there is a cell in adult marrow and in adult spleen that is capable of reacting against the tolerant host or against the lethally irradiated subject The result of this reaction is an immunological cripple

For reasons of supply we must use adult marrow to restore marrow function in individuals who have received lethal total-body irradiation—there simply is not enough fetal marrow available—and we must use cells of fetal spleen that are capable of tolerance to restore function in the lethally irradiated and unresisting lymphopoietic organs, that is, lymph nodes and spleen

Adult marrow is probably deficient in some species, and perhaps in man, in the number of cells of the "splenic" type that function as restorers of lymph nodes and spleen following radiative destruction of these organs Moreover, as Billingham and others have shown, the adult splenic-type cell in marrow reacts against the host in these irradiated organs Either way, from immunological warfare or from inadequate numbers of immigrant seed cells of lymphopoietic type, one comes up with an immunological cripple unable to defend itself against foreign invaders, including viruses, bacteria, yeasts, parasites, and homografts We see evidence of this crippling in our irradiated patients and in our dogs, in which, perhaps, our ability to make clinical observations somewhat exceeds that in small rodents

The point of this preamble is that I should like to know what cell have we in mind in the experiments Billingham has described so that we can remove it from adult marrow and, in tolerant form and adequate number from fetal spleen, add it to the infusions we give our patients

this increase continued up to the maximal interval tested (107 days) At this time the mean was still below that of the control (nonpreinjected) group FIGURE 2 shows the geometric mean peak titer of each group of recipients, these points being connected by the solid line The horizontal lines show the geometric mean titer of the control rabbits in the case of each interval

*Species Specificity*

In a number of experiments leukocytes obtained from the blood of other species of animals were tested It was found that leukocytes of horse, cow, and chicken blood did not affect the geometric mean agglutinin titer of recipients when preinjected in numbers equal to those of the rabbit leukocytes In the case of human leukocytes a partial effect was observed, the geometric mean titer being substantially lower than that of the controls (somewhat more than 2 powers of 2) However, this geometric mean peak titer was substantially higher than that found in the group preinjected with rabbit leukocytes These results are shown in TABLE 1 Also shown are data obtained with the preinjection of the same number of rabbit erythrocytes in which the leukocytes had

TABLE 1
PEAK AGGLUTININ TITERS OF RECIPIENT RABBITS PREINJECTED WITH VARIOUS BLOOD CELLS*

| Control rabbits (not preinjected) | Recipient rabbits preinjected with | | | | | |
|---|---|---|---|---|---|---|
| | Blood leukocytes of | | | | | Rabbit erythrocytes |
| | Rabbit | Man | Horse | Cow | Chicken | |
| 256 | 64 | 64 | 256 | | | 384 |
| | <12 | 16 | 64 | | | 384 |
| | <12 | | | | | 16 |
| 96 | 12 | | | | | 48 |
| 96 | <12 | | | | | 48 |
| | <12 | | | | | |
| 128 | <12 | 384 | | | | 512 |
| | <12 | 32 | | | | 192 |
| | 16 | 24 | | | | 32 |
| | | 24 | | | | |
| 192 | 12 | | | 384 | 384 | 768 |
| 192 | <12 | | | 192 | 384 | 384 |
| 768 | | | | 128 | 192 | 128 |
| 192 | | | | 128 | 128 | 96 |
| | | | | 96 | 24 | |
| 768 | <12 | 256 | 384 | | | 1024 |
| 384 | <12 | 64 | 384 | | | 512 |
| 128 | <12 | 16 | 256 | | | 384 |
| 96 | 12 | <12 | 128 | | | 96 |
| 64 | | | 96 | | | |
| Geometric mean titer of group ($\log_2$) 7 6 | 3 0 | 5 3 | 7 5 | 7 3 | 7 2 | 7 4 |

* Reproduced by permission from the *Proceedings of the Society for Experimental Biology and Medicine* [3]

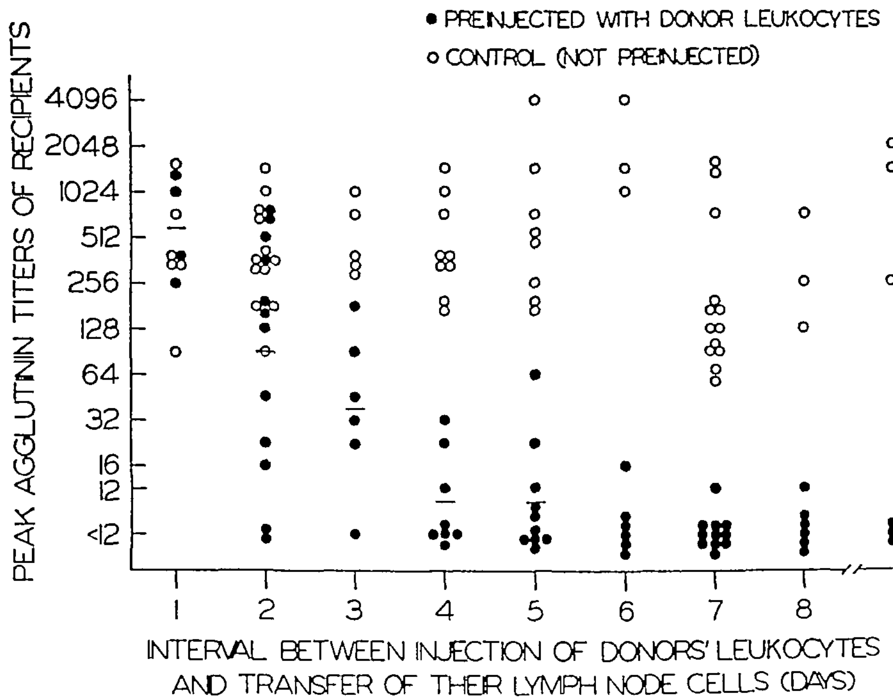



FIGURE 1 Peak agglutinin titers of individual recipients of lymph node cells incubated *in vitro* with filtrates of trypsin-treated suspensions of dysentery bacilli The recipients in the experimental group had been injected intradermally with donor leukocytes at intervals, as indicated, prior to transfer of the antigen-incubated lymph node cells The bar indicates the geometric mean titer of the group of preinjected recipients Reproduced by permission from the *Proceedings of the Society for Experimental Biology and Medicine* [3]

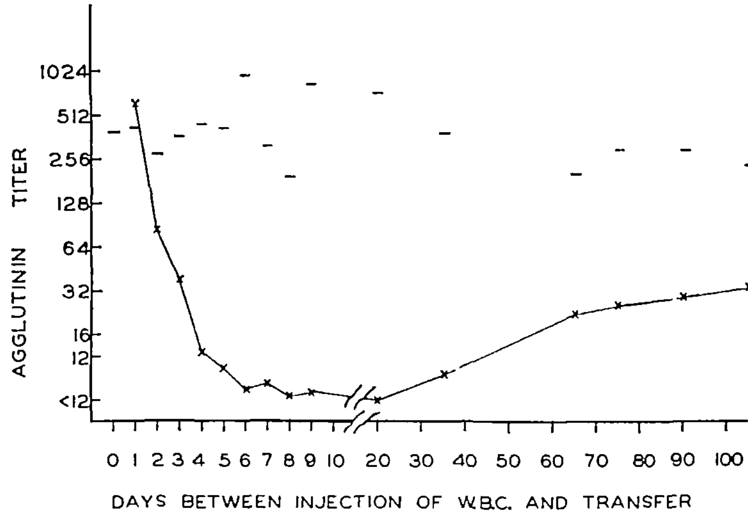



FIGURE 2 Geometric mean peak agglutinin titers of recipient rabbits that had been preinjected with leukocytes of other (donor) rabbits at various intervals prior to the transfer of antigen-incubated lymph node cells from the same donor rabbits Geometric mean peak titers of control (nonpreinjected) rabbits for each group are shown by the horizontal lines

this increase continued up to the maximal interval tested (107 days) At this time the mean was still below that of the control (nonpreinjected) group FIGURE 2 shows the geometric mean peak titer of each group of recipients, these points being connected by the solid line The horizontal lines show the geometric mean titer of the control rabbits in the case of each interval

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|---|---|---|---|---|---|---|
| | Blood leukocytes of | | | | | Rabbit erythrocytes |
| | Rabbit | Man | Horse | Cow | Chicken | |
| 256 | 64 | 64 | 256 | | | 384 |
| | <12 | 16 | 64 | | | 384 |
| | <12 | | | | | 16 |
| 96 | 12 | | | | | 48 |
| 96 | <12 | | | | | 48 |
| | <12 | | | | | |
| 128 | <12 | 384 | | | | 512 |
| | <12 | 32 | | | | 192 |
| | 16 | 24 | | | | 32 |
| | | 24 | | | | |
| 192 | 12 | | | 384 | 384 | 768 |
| 192 | <12 | | | 192 | 384 | 384 |
| 768 | | | | 128 | 192 | 128 |
| 192 | | | | 128 | 128 | 96 |
| | | | | 96 | 24 | |
| 768 | <12 | 256 | 384 | | | 1024 |
| 384 | <12 | 64 | 384 | | | 512 |
| 128 | <12 | 16 | 256 | | | 384 |
| 96 | 12 | <12 | 128 | | | 96 |
| 64 | | | 96 | | | |
| Geometric mean titer of group ($log_2$) 7 6 | 3 0 | 5 3 | 7 5 | 7 3 | 7 2 | 7 4 |

* Reproduced by permission from the *Proceedings of the Society for Experimental Biology and Medicine* [3]

been reduced to approximately 0 1 per cent It can be seen that recipients preinjected with such suspensions did not show lower geometric mean peak agglutinin titers than the controls (not preinjected)

### *Variation in Numbers of Leukocytes Preinjected and the Route of Injection*

In the experiments described thus far, the number of leukocytes preinjected intradermally was $10^7$ In other experiments the number of cells was varied, and the intravenous route was used as well No substantial differences were found between the effects of intravenous or intradermal preinjection of leukocytes over the range of $10^8$ to $10^5$ cells In either case, in the recipients of the lymph node cells preinjection of $10^8$ or $10^7$ was followed by agglutinin titers near or below the threshold of measurement, following preinjection of $10^6$ leukocytes the titers of the recipients were quite low, but higher than those referred to above and, following preinjection of $10^5$ leukocytes, these titers were still higher, but well below the means found in the nonpreinjected controls These results are shown in FIGURE 3

### *Individual Donors of Leukocytes*

In all cases in which the same group of rabbits was used as a source both of pooled leukocytes for preinjection and of lymph node cells for transfer there was complete "suppression" of the cell-transfer effect, that is, there was almost no measurable agglutinin titer in the recipients subsequent to cell transfer The same results were obtained when the leukocytes for preinjection were pooled from one group of rabbits and the lymph node cells from another group In a number of experiments individual rabbits were used as the source of leukocytes for preinjection, and other individual rabbits were used as sources of lymph node cells In the case of 20 recipients in such experiments, 16 were

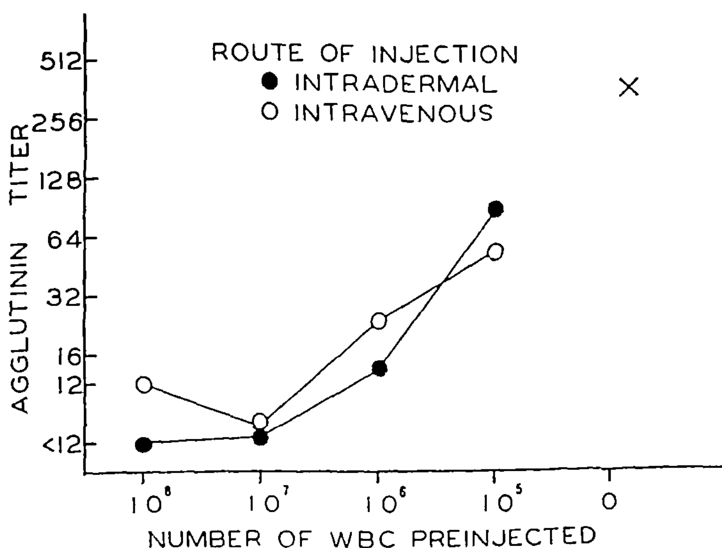



FIGURE 3 Geometric mean peak titers of lymph node cell recipients preinjected with donor leukocytes in various numbers intradermally or intravenously The X (upper right) represents the mean titer for nonpreinjected controls

TABLE 2

MAXIMUM AGGLUTININ TITERS OF RECIPIENTS PREINJECTED WITH LEUKOCYTES OF INDIVIDUAL DONORS AND GIVEN INCUBATED LYMPH NODE CELLS FROM OTHER DONOR RABBITS

| Donors of | | Peak agglutinin titers of individual recipients | |
|---|---|---|---|
| Leukocytes for preinjection | Lymph node cells for transfer | Preinjected | Not preinjected |
| A | K | <12 <12 | |
| B | L | <12 <12 | 384 |
| C | M | 32 1024 | 768 |
| D | N | 24 24 | 1536 |
| E | O | <12 32 | 1536 |
| F | P | 16 24 | |
| G | Q | 64 192 | |
| H | R | 12 <12 | |
| I | S | <12 384 | |
| J | T | <12 <12 | |
| Pool of above | Pool of above | <12 <12 <12 | |
| Albino | Chinchilla | <12 <12 24 | |
| | | <12 <12 | |
| Albino | Albino | <12 384 | 128 |
| | | | 512 |
| | | | 512 |
| Chinchilla | Albino | <12 192 | |
| | | 128 4096 | |
| Chinchilla | Chinchilla | <12 <12 | |

found to have low titers, at or near the limit of detection, the titers of 3 others were in an intermediate range, and the titer of one was quite within the range of the control (nonpreinjected) animals These data are shown in the upper part of TABLE 2 In the lower half of the table are shown the results of experiments involving albino and chinchilla rabbits As can be seen, no consistent difference between rabbits of these two groups was demonstrated

*Preinjection of Recipients with Their Own Leukocytes or with Leukocytes of Other Rabbits*

The effect of preinjection of a recipient's own leukocytes, in comparison with those of other rabbits, was studied by the use of leukocyte suspensions injected intradermally or intravenously and by means of whole blood reinjected intravenously In each case recipients other than the one bled were also injected with the leukocytes, and one week later all of the recipients were given antigen-incubated lymph node cells pooled from a group of donor rabbits The peak agglutinin titer for each recipient was determined, and the geometric mean for each experimental group is shown in TABLE 3 It can be seen that the geometric mean titers of the groups preinjected with their own leukocytes were in the same range as, but somewhat lower than, those of the control groups, whereas the groups injected with suspensions of leukocytes from other rabbits showed substantially lower mean titers In the case of whole blood, in an amount containing the same number of leukocytes, the mean titer of recipients

TABLE 3

PREINJECTION OF EACH RECIPIENT WITH ITS OWN LEUKOCYTES OR WHOLE BLOOD OR WITH LEUKOCYTES OF ANOTHER RABBIT

| Preparation | Route of injection | Mean peak agglutinin titers of recipients preinjected with | | | | | |
|---|---|---|---|---|---|---|---|
| | | Own leukocytes | | Leukocytes of other rabbits | | Controls (nonpreinjected) | |
| | | No of rabbits | Mean titer | No of rabbits | Mean titer | No of rabbits | Mean titer |
| Leukocyte suspension | i d | 18 | 7 0 | 17 | 4 7 | 11 | 7 7 |
| Leukocyte suspension | i v | 26 | 7 3 | 15 | 4 2 | 12 | 7 5 |
| Whole blood | i v | 21 | 8 2 | 13 | 5 4 | 11 | 7 7 |

Mean titers expressed as $\log_2$

preinjected with their own blood was slightly higher than that of the nonpreinjected controls, the mean titer of recipients preinjected with blood of other rabbits showed some reduction in titer, but not as much as in the case of preinjection with leukocytes

*Effects of Preinjection of Leukocytes Treated in Various Ways*

Leukocytes were obtained from the blood of prospective donors of lymph node cells and then were treated in various ways before preinjection into the prospective recipients Comparisons were made in each case between the effectiveness of preinjection of such treated cells and that of untreated portions of the same leukocyte suspension in causing reduction of the agglutinin titers of the recipient rabbits The majority of the forms of treatment carried out markedly reduced the effectiveness of the leukocyte suspension, so that the titers of the corresponding recipients showed a range and mean value quite similar to those of the nonpreinjected controls These treatments were as follows heating for 60 minutes at 60° C, alternate freezing in a dry-ice and alcohol bath at −70° C and thawing, suspension of the cells in distilled water, addition of sodium iodoacetate to $10^{-2}$ M, and lyophilization Two other treatments did not alter the effectiveness of the leukocytes X irradiation at a dose of 400 r, and disruption in a magnetostriction oscillator In the latter case the suspension of disrupted cells was centrifuged for testing of the supernate and resuspended sediment separately It was found that the supernate was without effect, but that the resuspended sediment was nearly as effective as the original suspension in causing reduction of the agglutinin titers

*Discussion*

The data presented above indicate that the results of lymph node cell transfer can be affected substantially by the prior injection of blood leukocytes from the donors of the lymph node cells if the leukocytes are injected at a sufficient interval before the cell transfer and in sufficient numbers relative to this

interval Such an effect, as indicated by lower concentrations of agglutinins to *Shigella* in the sera of recipient animals, was demonstrated not only with leukocytes pooled from groups of rabbits that were to be used as the donors of the transferred lymph node cells, but also with leukocytes from groups of rabbits other than the lymph node cell donors The effect was demonstrable even with leukocytes from individual rabbits, either the prospective donors of the transferred lymph node cells or others The difference in observed events subsequent to the transfer of lymph node cells according to whether the recipients are encountering cells from the donor for the first or the second time (after preinjection) is analogous to the accelerated rejection of skin grafts placed for a second time from a given donor to a given recipient, the "second-set phenomenon" described by Medawar[4, 5] and since then studied by several other workers [6] There is an apparent difference between the two situations in that the rejection of second-set skin homografts involves a difference of time, in comparison with their controls, whereas in the present system antibody fails to appear altogether In all probability this difference is due merely to the fact that there are differences in the respective experimental conditions Thus, in the case of skin grafting the transplanted tissue must await vascularization for full contact with the blood of the recipients, whereas in lymph node cell transfer the transplanted cells are introduced directly into the blood by intravenous injection

The following five observations made in this study are analogous to the corresponding observations reported in studies of skin homografting

(1) A definite minimum interval of time between leukocyte preinjection and lymph node cell transfer was found necessary in this study for the suppressive effect on the transferred cells, and a certain minimum of time must elapse between first and second skin homografts in order to elicit the second-set phenomenon [5]

(2) The suppressive effect on transferred lymph node cells (indicated by decreased recipient agglutinin titers) has been found in this study as long as 107 days after the preinjection of leukocytes, in mice, the second-set phenomenon has also been elicited as long as 120 days after the first skin graft [7]

(3) In this study the degree of suppression of activity of transferred lymph node cells was found, within a given range, to be a function of the number of leukocytes preinjected, and it has been reported that the survival time of skin homografts bears an inverse relationship to the dose of grafted skin employed [5]

(4) No evidence was obtained, in the present study, of common antigens between leukocytes and erythrocytes of the rabbit In skin homograft studies Medawar[8] had found that the second-set phenomenon could be induced not only by prior skin graft between the animals involved, but also by the injection of leukocytes, the injection of erythrocytes did not, however, have a similar effect Thus the antigens of rabbit leukocytes, at least those shared with skin, were found not to occur in erythrocytes as well

(5) The effects of certain treatments of the cells injected for the primary contact of donor and recipient have shown similarities between the two systems Of the treatments of preinjected leukocytes found in this study to inactivate

the effect several had been applied to rabbit leukocytes in skin grafting studies with analogous effects, that is, failure of induction of the second-set phenomenon These are heating, lyophilization, and freezing [9] Of the two treatments found not to inactivate the preinjection effect—X irradiation and sonic oscillation—the latter had been found not to inactivate rabbit leukocytes for induction of the second-set phenomenon [10] Also, the active material had been found in both systems to be present in the insoluble sediment of the oscillated cell suspension [10]

One observation made in these studies is at variance with a parallel observation reported in the skin homografting studies In preparing rabbits for accelerated rejection of skin grafts by injection of leukocytes, Medawar found intradermal injections of the latter to be 18 times as effective as intravenous ones [8] Later, Billingham and Sparrow reported that rabbits injected intravenously with suspensions of homologous, dissociated, viable epidermal cells (1 to 15 million) failed to show accelerated skin graft rejection, and in some of the experimental animals the survival of the graft was increased by a factor of 2 or 3 [11] In the present study no difference was found between these 2 routes of injection in the effectiveness of preinjected leukocytes, in the range between $10^6$ and $10^8$, as shown in TABLE 2 It may be mentioned in this connection that in the case of purebred strains of mice Billingham *et al* found it possible to induce accelerated skin graft rejection by the intravenous injection of leukocytes [12]

As in the case of skin grafting and other instances of tissue homotransplantation, there is evidence that this "preinjection effect" is immunological in nature First, the intervals between the preinjection of leukocytes and the lymph node cell transfer that are required for the partial and the complete effect, respectively, are consistent with the time after injection of cellular antigens at which one could expect the appearance of significant and maximal titers of antibody (to the 2-day and 6-day intervals indicated in TABLE 1 and FIGURE 1 for the partial and complete effects, respectively, on the transferred cells, should be added 3 days, the interval after transfer of such cells after which the anti-*Shigella* antibody appears) Further evidence of an immunological mechanism based on rabbit leukocyte antigens is offered by the species specificity of the reaction, that is, that the preinjection of leukocytes of several mammalian species other than the rabbit failed to affect the results of subsequent transfer of rabbit lymph node cells Finally, an immunological mechanism is suggested by the failure of preinjection of the recipient's own leukocytes to affect substantially its subsequent agglutinin titer The data in TABLE 3 which show this are of interest in another connection also As can be seen in that table, the intradermal or intravenous preinjection of the recipient's own leukocytes, in a prepared suspension, caused a slight decrease in titers in comparison with the nonpreinjected controls, the average decrease in geometric mean titer being about one half a power of 2 It was considered possible that manipulation of the blood in concentrating the leukocytes could have caused enough denaturation of leukocyte proteins to have rendered some of these slightly antigenic to the rabbit from which the blood was drawn The results

of experiments with whole blood in amounts containing the same numbers of leukocytes supported this possibility, since the preinjection of the recipient's own blood caused no reduction in mean titer The preinjection of blood of other rabbits did cause a reduction of the mean agglutinin titer of the recipient rabbits, but a smaller difference than that found in recipients preinjected with prepared suspensions of leukocytes of other rabbits These data again support the conclusion that the effects of preinjection of leukocytes are largely due to differences in antigenic structure in leukocytes among members of the species, but that a small part of the effect of leukocyte preinjection on the lymph node cell transfer observed throughout this study could be due to changes in leukocyte protein per se

Consequently, the effect of preinjection of leukocytes on subsequent lymph node cell transfer would, then, appear to rest on individual tissue specificity within members of the rabbit species This interpretation would also be consistent with the finding that substantial preinjection effects were obtained invariably in the case of leukocytes pooled from several rabbits, and sporadically in the case of leukocytes from other, individual rabbits Data from the experiments done by preinjecting leukocytes from other individual rabbits are of interest from the viewpoint of the random distribution of the individual antigens involved in tissue specificity Experiments of this kind, shown in TABLE 2, involved the preinjection of leukocytes from 1 rabbit to 2 recipients and the transfer to the latter of lymph node cells from another individual rabbit Possible immunological relationships that could exist among these animals and that would determine the outcome of such experiments include the following If the donor of the preinjected leukocytes and that of the lymph node cells have at least one common antigen that the recipient does not have, and to which it is therefore able to react immunologically, one could expect a substantial degree of "suppression" of the transferred cells (that is, a reduction in subsequent agglutinin titer of the recipient) On the other hand, if the two donor rabbits have no common antigen, or if they have common antigens but the recipient also has any antigen common to both donors and is thus unable to react immunologically to it, one would expect no suppression of the cells Intermediate recipient agglutinin titers, indicating partial suppression of the transferred cells, would reflect degrees of effective antigenicity of the tissue factors, perhaps on the basis of smaller differences in configuration between antigenic groupings of the tissue factors involved In a random population, such as the rabbits used here, it would therefore be possible to encounter any combination of results in experiments such as those shown in TABLE 2 The fact that, of 20 recipients shown there as receiving the preinjected and the transferred cells from 2 different individual rabbits, 16 had markedly reduced titers, suggests that the total number of individual tissue antigens in leukocytes of rabbits is considerable, and that each animal carries several of these, so that the probability of a common antigen in two rabbits chosen at random would be high, but that each animal has substantially less than one half the total number, so that the probability that a randomly chosen recipient would not have the same antigens would again be high

*References*


1. HARRIS, S , T N HARRIS & M B FARBER 1954 J Immunol **72** 148
2. HARRIS, S , T N HARRIS, C A OGBURN & M B FARBER 1956 J Exptl Med **104.** 645
3. HARRIS, T N , S HARRIS & M B FARBER 1957 Proc Soc Exptl Biol. Med **95.** 26
4. MEDAWAR, P B 1944 J Anat **78** 176
5. MEDAWAR, P B 1945 J Anat **79:** 157
6. ANN N Y ACAD SCI 1957 64(5) 735–1073
7. BILLINGHAM, R E , L BRENT & P B MEDAWAR 1954 Proc Roy Soc. London. **B143** 58
8. MEDAWAR, P B 1946 Brit J Exptl Pathol **27.** 15
9. MEDAWAR, P B *In* Preservation and Transplantation of Normal Tissues 1 Ciba Foundation Symposium Little, Brown & Co Boston, Mass
10. BILLINGHAM, R E , L BRENT & P B MEDAWAR 1956 Nature **178** 514
11. BILLINGHAM, R E & E M SPARROW 1955 J Embryol Exptl Morphol **3.** 265
12. BILLINGHAM, R E , L BRENT & P B MEDAWAR 1956 Transplantation Bull **3** 84

# TOLERANCE AND HOMOLOGOUS DISEASE IN IRRADIATED MICE PROTECTED WITH HOMOLOGOUS BONE MARROW*

*By* John J. Trentin
*Departments of Anatomy, Baylor University College of Medicine and University of Texas Dental Branch, Houston, Texas*

The protection of mammals of several species against otherwise lethal doses of whole-body X irradiation by postirradiation transfusion of bone marrow is a well-established phenomenon Protection is associated with reseeding and repopulation of the hematopoietic system with cells of marrow-donor origin, regardless of whether protection is achieved with isologous (same genotype), homologous (different genotype, same species), or heterologous (different species) bone marrow[1] Protection with homologous or heterologous bone marrow confers tolerance of skin grafts of marrow-donor antigenic type (that is, from the marrow donor or its parent strains if the marrow donor is an $F_1$ hybrid), as well as of the transplanted marrow[2-5] Mice protected with isologous marrow are not rendered tolerant of homologous skin[2, 3, 5] Mice protected with homologous marrow may or may not show tolerance of skin from a third homologous strain[5] Mice protected with rat marrow may accept rat skin while simultaneously rejecting homologous mouse skin[4]

TABLE 1 presents the results of an experiment designed to determine the lowest dose of whole-body X irradiation, followed by marrow from an $F_1$ hybrid, that would result in tolerance of skin homografts from the foreign parent strain[6] Only at the high dose levels of $LD_{90}$ and above was tolerance observed At the high sublethal dose of 550 r, skin grafting could not be performed, since most of the mice died within 2 or 3 weeks after irradiation The same dose of homologous $F_1$ marrow essential for survival after a lethal dose of irradiation resulted in death when given after a high sublethal dose of irradiation[1] Death was preceded by a precipitous loss of body weight and reduced circulating red and white blood cell levels as compared to mice receiving the same marrow after 770 r Evidence will be presented later indicating that this high sublethal zone mortality is the result of recovery of the ability of the host to reject the temporarily grafted homologous marrow Main and Prehn[5] found that 96 per cent of irradiated mice accepted skin homografts from the marrow donor after 600 r and 29 per cent after only 300 r It is probable that the difference in results is related to the difference in genetic material used The skin donor and recipient strains of TABLE 1 are of different histocompatibility-2 (H-2) genotype Main and Prehn used two strains of the same H-2 genotype The H-2 locus is the strongest of the loci determining histocompatibility in the mouse[7]

What is the nature or mechanism of the tolerance of homologous skin induced by irradiation protection with bone marrow of skin donor type? To use the terms of Billingham *et al*,[8] is it the result of a central failure, or of a peripheral failure (afferent or efferent), of the immunological response that constitutes the

* The work reported in this paper was supported in part by Research Grant C-3367 from the National Cancer Institute, Public Health Service, Bethesda, Md

TABLE 1
MORTALITY AND SKIN TRANSPLANTABILITY IN IRRADIATED AND MARROW-TREATED CBA MICE

| X-ray dose r | No marrow | | (Cb X CBA) $F_1$ marrow | | | | |
|---|---|---|---|---|---|---|---|
| | No of Mice | 30 day % mortality | No of mice | 30 day % mortality | No grafted with Cb skin | No of takes | No of sloughs |
| 0 | | | 12 | 0 | 12 | 0 | 12 |
| 110 | | | 12 | 0 | 12 | 0 | 12 |
| 330 | 12 | 0 | 36 | 0 | 12 | 0 | 12 |
| 550 | 23 | 13 | 36 | 89 | 0 | | |
| 600 | 12 | 50 | | | | | |
| 660 | 48 | 90 | 12 | 17 | 9* | 6 | 0 |
| 770 | 60 | 100 | 72 | 10 | 23 | 20 | 3 |

* Three mice dead with viable grafts in 13 to 18 days

homograft reaction? To test for the possibility of a central failure by the technique of Billingham *et al*,[9] lymphoid tissue from normal nonimmune CBA mice was injected into 9 CBA mice bearing successful homografts of Cb† skin (162 to 182 days' duration) as a result of 770 r of X irradiation followed by intravenous transfusion with (Cb X CBA) $F_1$ hybrid marrow 36 to 56 days prior to skin grafting[10] Partial or complete breakdown of the skin graft occurred in all cases and was accompanied by anemia, hemorrhage, and mortality in several of the mice, which was indicative of rejection not only of the skin homograft, but also of the "borrowed" homologous bone marrow on which these mice were dependent for continued existence (FIGURES 1 and 2) The results indicate a central failure or absence of immunological response as the reason for the tolerance induced by irradiation protection with homologous bone marrow The results, however, do not answer the question of whether the "central failure" is the result of (1) an altered immunological specificity, in the sense of Billingham *et al*,[9] on the part of any surviving antibody-producing tissue of the irradiated host, or (2) destruction of the antibody-producing tissue of the host, with substitution thereof by the antibody-producing tissue (and immunological specificity) of the homologous marrow donor

The genetic relationship between marrow donor and lethally irradiated recipient is of great importance with respect to degree of initial protection, duration of survival, and induction of tolerance[3, 11] Mice protected with isologous marrow show long-term survival and are not rendered tolerant of homologous skin grafts[2, 3] Mice protected with homologous or heterologous marrow or spleen usually show irradiation recovery followed by a secondary loss of weight frequently resulting in death during the second month postirradiation, without secondary hematopoietic depression[3, 11-13] Delayed mortality with heterologous marrow is more severe than with homologous marrow Delayed mortality with homologous marrow is more severe when the foreign-strain marrow is from a donor of different H-2 genotype (A into C57, Dba into A, A into

† Cb = BALB/c

FIGURE 1 CBA mouse bearing successful homograft of Cb skin of 181 days' duration as a result of protection against the otherwise lethal dose of 770 r by postirradiation transfusion of (Cb × CBA) $F_1$ hybrid bone marrow 36 days before skin grafting Photograph taken at time of injection with normal CBA lymphocytes For result, see FIGURE 2

FIGURE 2 Same mouse as in FIGURE 1, 21 days after intraperitoneal and subcutaneous injection of suspension of lymph node and spleen cells recovered from one normal unirradiated CBA mouse Rejection of the skin homograft has begun and went to completion during the ensuing 12 days

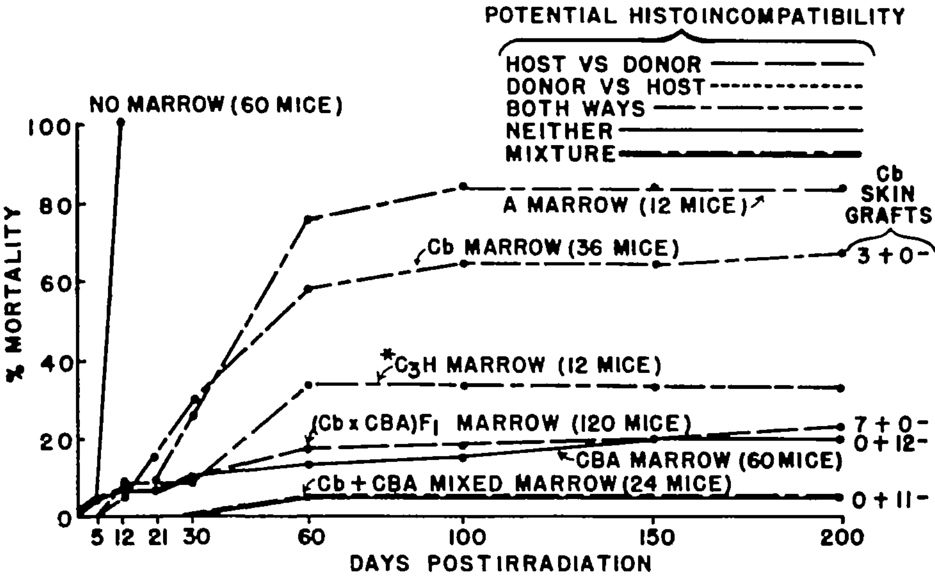



FIGURE 3 Cumulative mortality and skin transplantability in CBA mice receiving 770 r whole-body X irradiation followed by indicated marrow treatment

* Of 6 mice grafted, 4 accepted C3H skin, and 2 accepted CBA skin

CBA, or Cb into CBA) than when it is from a donor of the same H-2 genotype (C3H into CBA) as the recipient (FIGURE 3)[3]

Most investigators early assumed,[12, 14] and some still believe[15] that this delayed mortality after good early protection against otherwise lethal irradiation is the result of recovery of the host's ability to reject the grafted homologous or heterologous marrow elements However, several facts do not support this hypothesis[3] There is usually no secondary hematopoietic depression at the time of late death[3, 13] If death results from rejection of the grafted marrow, one should see evidence of this, as in the above-mentioned high-sublethal zone mortality, and in the abolition of the tolerant state by injection of host-type lymphocytes Morever, the duration of the induced tolerance of homologous skin after protection against lethal levels of irradiation by homologous marrow is of very long or indefinite duration, and such skin homografts or heterografts are usually in good condition at the time of "delayed death"[3, 4] (FIGURE 4) Because of this and the usual lack of characteristic secondary disease with isologous marrow, or with $F_1$ hybrid homologous marrow in an irradiated parent strain, it was suggested that the secondary disease was more likely the result of reaction of graft against host than host against graft[3] It is well established that the homograft reaction is an immunological reaction to foreign tissue antigens, and that the lymphoid system plays an important role in its mediation Skin homografts usually become vascularized during the first week, as do isografts During the second week, however, the homograft reaction sets in, resulting in vascular breakdown and sloughing A second homograft from the same source breaks down more rapidly than the first[16] This "immunity" may be passively transferred by the regional lymph nodes of an animal that has recently rejected a first graft[16, 17] The homograft reaction may be delayed



FIGURE 4 (Cb × CBA) $F_1$ hybrid mouse dead of homologous disease 48 days after protection against the otherwise lethal dose of 770 r by postirradiation transfusion of Strain A bone marrow Homograft of Strain A skin of 41 days' duration is in excellent condition at time of death from homologous disease

by factors such as cortisone and X irradiation, which suppress antibody formation[7] The homograft reaction is absent or weak in humans with agammaglobulinemia[18] It is also well established[7] that an $F_1$ hybrid of two inbred strains of mice will accept tissue transplants from either parent strain, the histocompatibility antigens being determined by dominant genes The $F_1$ hybrid, inheriting all of the histocompatibility antigens of both parents, cannot react against tissues from either parent However, each parent strain rejects tissue grafts from the $F_1$ hybrid, each reacting against the foreign tissue antigens of the other parent in the $F_1$ hybrid Combinations of $F_1$ tissue into irradiated parent strains and vice versa therefore constitute "one-way streets," immunologically speaking In the former case only reactions of the host against the graft are potentially possible In the latter case only reactions of the graft against the host may occur (and that only if lymphoid elements or their precursors have been transplanted) As such, these combinations constitute ideal systems for resolving the questions of whether the two types of mortality referred to above are the result of reactions of the host against the graft or of the graft against the host Results of such experiments are shown in FIGURES 3 and 5 to 8 and are summarized in TABLE 2 Some of these data have been published previously[19]

Early mortality (5 to 21 days) after the otherwise high sublethal dose of 550 r

occurred only in those combinations where the recipient strain can potentially reject tissue grafts from the donor strain, regardless of whether (Cb into CBA) or not ($F_1$ into CBA) the donor strain can react immunologically against the recipient strain. It was not seen in those combinations where the recipient strain could not reject grafts from the donor strain (CBA into CBA, $F_1$ into $F_1$, CBA into $F_1$, and Cb into $F_1$) even though the donor strain can react immunologically against the recipient strain in the latter two combinations.

At 770 r no early mortality with secondary hematopoietic depression was seen in any combination. Delayed mortality (21 to 60 days) after good 21-day protection against the otherwise lethal dose of 770 r was seen with A marrow into CBA, Cb into CBA, C3H into CBA (slight), A into (Cb × CBA) $F_1$,

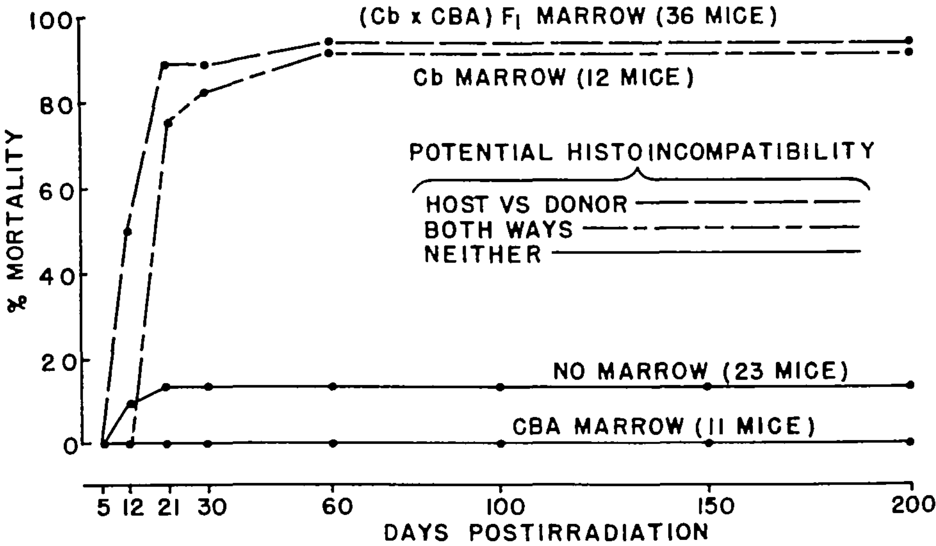



FIGURE 5. Cumulative mortality and skin transplantability in CBA mice receiving 505 r whole-body X irradiation followed by indicated marrow treatment.

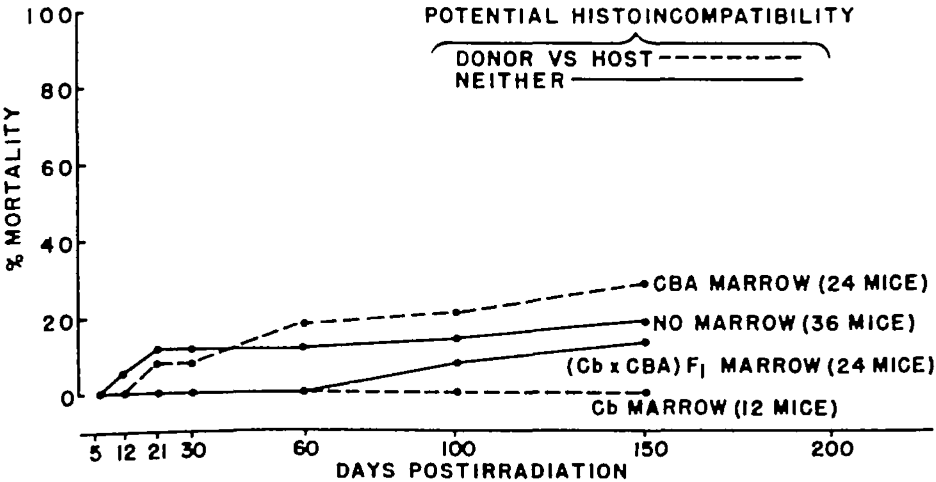



FIGURE 6. Cumulative mortality and skin transplantability in (Cb × CBA) $F_1$ hybrid mice receiving 550 r whole-body X irradiation followed by indicated marrow treatment.

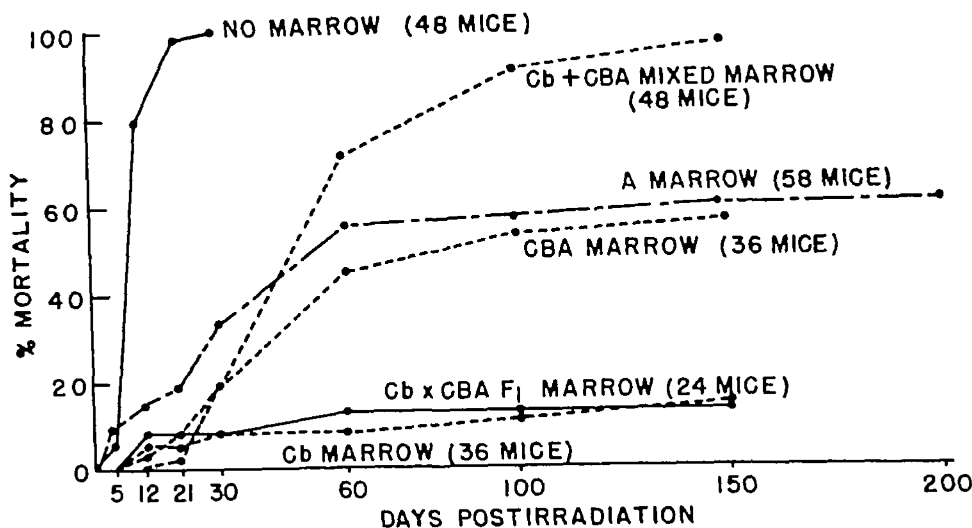



FIGURE 7 Cumulative mortality and skin transplantability in (Cb × CBA) $F_1$ hybrid mice receiving 770 r whole-body X irradiation followed by indicated marrow treatment

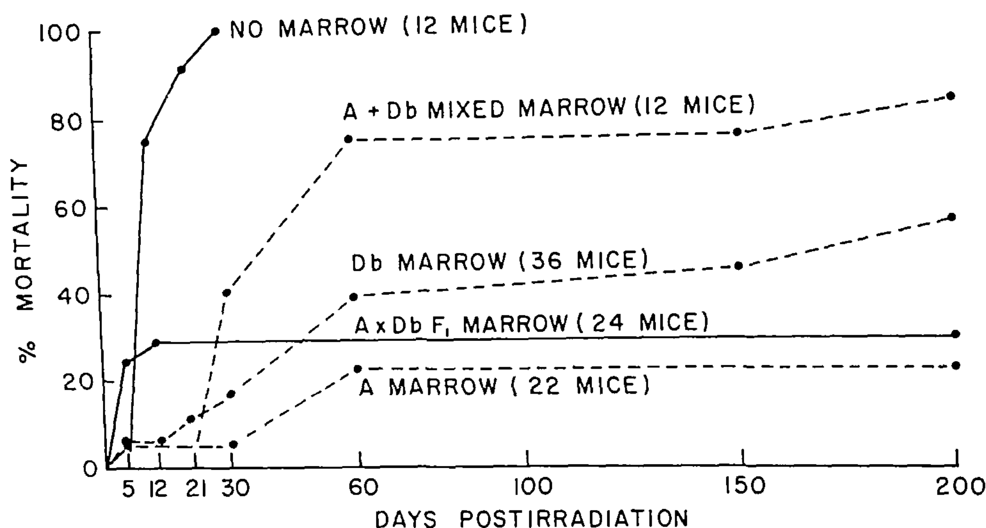



FIGURE 8 Cumulative mortality and skin transplantability in (A × Db) $F_1$ hybrid mice receiving 770 r whole-body X irradiation followed by indicated marrow treatment

Cb plus CBA into (Cb × CBA) $F_1$, CBA into (Cb × CBA) $F_1$, A plus Db into (A × Db) $F_1$, Db into (A × Db) $F_1$, and A into (A × Db) $F_1$ (slight) That is, it occurred only where the donor strains can react immunologically against the recipient strain, even though in the latter five combinations the recipient strain cannot react against the donor strains The only combination of parent marrow into $F_1$ hybrid that failed to give delayed mortality after 770 r was Cb into (Cb × CBA) Delayed mortality in lethally irradiated $F_1$ hybrids protected with parent strain marrow has also been reported by Uphoff[20] for other combinations of parent strains However, van Bekkum *et al* [11] and Schwartz *et al* [21] failed to obtain delayed mortality with parent marrow into irradiated $F_1$ hybrids for still other combinations of parent strains It would appear, therefore, that with parent strain marrow into $F_1$ hybrid after an other-

TABLE 2

SUMMARY OF THE RESULTS SHOWN IN FIGURES 3 AND 5 TO 8, CORRELATING THE TWO TYPES OF MORTALITY WITH THE COMBINATIONS OF HOST AND MARROW-DONOR STRAINS IN WHICH EACH WAS OBSERVED, AND THE IMMUNOLOGICAL POTENTIALS OF THE HOST AND DONOR STRAINS AGAINST EACH OTHER

| | Isologous | Homologous combinations | | |
|---|---|---|---|---|
| | Neither host nor donor capable of histocompatibility reaction against each other | Both host and donor capable of reacting against each other | Host potentially capable of reacting against donor, but not vice versa | Donor potentially capable of reacting against host, but not vice versa |
| | Same genotype | Unrelated strains | $F_1$ marrow into parent | Parent marrow into $F_1$ hybrid |
| 5- to 21-day mortality after otherwise sublethal irradiation | − | + | + | − |
| 21- to 60-day mortality following early protection against otherwise lethal irradiation | − | + | − | + or − |

wise lethal dose of irradiation, delayed mortality may or may not occur, depending on the particular combination of strains used. In those $F_1$ hybrids that do not show delayed mortality after parent marrow, there may nevertheless be body weight loss, indicating that secondary disease may be present, but sublethal.[19] Important factors in determining the severity of secondary disease are probably the degree of antigenic difference between the strains of mice used and the degree of lymphoid regeneration from the grafted marrow. Data indicating lymphoid regeneration from marrow donor origin have been obtained by several laboratories,[22-26] and Mitchison[27] has demonstrated the production of antibodies by spleen cells transplanted from immunized donors into irradiated homologous mice.

At 770 r, delayed mortality was not seen with (Cb × CBA) $F_1$ marrow into the CBA parent, even though the recipient strain can potentially reject tissue grafts from the donor strain, and even though this same combination gave high mortality of the early type after sublethal irradiation. It would appear, therefore, that "early mortality" after sublethal irradiation is a "grafted-marrow-rejection mortality" related to recovery of the ability of the host to reject the temporarily transplanted homologous marrow elements, whereas "delayed mortality" after otherwise lethal irradiation is the result of the transplanted marrow (or its lymphoid derivatives) "rejecting" the tolerant host. This is in accord with the observed secondary hematopoietic depression in the case of "early mortality" and the lack of it in the case of "delayed mortality."

A sublethal or midlethal zone effect has been observed also by Congdon *et al.*[28] in mice receiving rat marrow and by van Bekkum *et al.*[11] in mice receiving either rat marrow or foreign-strain mouse marrow, but not isologous marrow. Congdon *et al.* nicely demonstrated a secondary degeneration of the transplanted bone marrow and were able to prevent mortality by administering

isologous marrow 6 days after the rat bone marrow However, they infer that because this type of mortality results from reaction of the host against the graft, the delayed mortality following protection against lethal irradiation with heterologous or homologous marrow is probably also caused by an immune reaction of host against graft [15,28] This is not in accord with the usual lack of marrow depression in delayed mortality,[13] or with the results obtained with $F_1$ marrow into parent and parent marrow into $F_1$ mice, neither is it in accord with the long duration of tolerance to skin grafts of marrow donor origin and the usually good condition of such skin homografts or heterografts at the time of delayed mortality[3,4] or with the findings of Weyzen and Vos[29] and of Grabar *et al*[30] that the serum gamma globulin in mice protected against lethal irradiation with rat bone marrow is of rat origin

The dose of irradiation is a very important determinant of the type of mortality resulting from subsequent administration of homologous or heterologous marrow Doses of 330 r and below permit homograft rejection, but without mortality (TABLE 1) In the high sublethal and midlethal dose range, a high mortality rate attends the rejection of homografted marrow Above the $LD_{100}$, tolerance of the grafted marrow usually ensues and the predominant cause of delayed mortality is homologous or heterologous disease, attributable to an immunological reaction of the graft against the host It is probable that between the latter two dose ranges both types of mortality (host versus graft and graft versus host) occur in the same population when heterologous or foreign strain homologous marrow is used Only by using the unique combinations of parent marrow into $F_1$ and vice versa can the two types of mortality be clearly dissociated at all dose levels

The terms "homologous disease" and "heterologous disease" have been proposed to designate the specific complications and mortality resulting from the successful transplantation of homologous or heterologous, as opposed to isologous or autologous, bone marrow or lymphoid tissue into a tolerant host, whether irradiated or not [19] Similar mortality has been observed in unirradiated animals rendered tolerant by transplantation of homologous spleen into the embryo or neonatal individual [31,32] If the graft-versus-host theory of the etiology of homologous disease is correct, similar mortality should occur also in unirradiated $F_1$ hybrids grafted with lymphoid tissue from either or both parent strains, since the $F_1$ hybrid, by genetic determination, is tolerant of grafts from the parent strains To test this hypothesis, 21-day-old (C57 × A) $F_1$ hybrids were given the equivalent of one spleen and mesenteric lymph node from either or both parent strains, or from another $F_1$ hybrid of the same sex The tissue was minced, suspended in saline, and injected intraperitoneally Controls received an injection of saline solution Body weights were recorded daily Lymphoid tissue from the A strain parent caused severe loss of body weight and death of most of the $F_1$ recipients within 60 days, with atrophy of the lymph nodes and thymus (TABLE 3) The body weight of the 60-day survivors was significantly below that of the saline controls, and a few died beyond 60 days Lymphoid tissue from the C57 parent had only a slight effect, and one half of the dose of tissue from each of both parent strains combined had an intermediate effect No such body weight loss or mortality was observed with

TABLE 3
MORTALITY IN UNIRRADIATED (C57 × A) $F_1$ HYBRIDS RECEIVING INDICATED TREATMENT AT WEANING AGE

| | Donor strain | No of mice | Per cent dead within 60 days | Mean survival (days) | Body weight difference from saline controls (grams) | |
|---|---|---|---|---|---|---|
| | | | | | At death | Survivors at 60 days |
| Spleen and lymph node | C57 | 21 | 5 | 16 | −2 2 | −0 1 |
| | A | 21 | 71 | 21 | −5 2 | −4 3 |
| | C57 and A | 32 | 28 | 28 | −4 4 | +0 4 |
| | (C57 × A)$F_1$ | 20 | 0 | | | −0 7 |
| Saline control | | 32 | 0 | | | Control |
| Bone marrow | A | 14 | 0 | | | +0 4 |
| | C57 and A | 31 | 0 | | | +0 3 |

isologous $F_1$ lymphoid tissue injections In contrast to the results after lethal irradiation protection, bone marrow from the parental strains into the unirradiated $F_1$ hybrid failed to result in weight loss or mortality, probably because of failure to multiply and differentiate into lymphoid elements in the unirradiated host Schwartz *et al*[21] have reported a fatal reaction caused by implantation of parental spleen tissue into sublethally irradiated $F_1$ hybrids, which they interpret as resulting from reaction of graft against host

*Summary*

Protection of mice against otherwise lethal doses of whole-body irradiation by means of homologous bone marrow results in tolerance of skin grafts of marrow donor antigenic type, as well as tolerance of the grafted marrow elements

The minimum dose of irradiation (followed by homologous marrow) necessary to produce such tolerance, using strains of different H-2 genotype, lies between an $LD_{13}$ and $LD_{90}$

Such tolerance results from a central failure or absence of the immunological response that constitutes the homograft reaction, and can be abolished by injection of unirradiated lymphoid tissue of host type

Two distinct types of mortality have been observed following homologous bone marrow transfusion into irradiated mice (1) "early mortality" (5 to 21 days) after an ordinarily high sublethal dose of irradiation, and (2) "delayed mortality" (21 to 60 days) following good early protection against an otherwise lethal dose of irradiation The former type is associated with secondary hematopoietic depression before death and occurs only in those combinations of strains in which the host strain can reject tissue grafts from the donor strain, regardless of whether the reverse is true This type of mortality, it is concluded, results from recovery of the host's ability to reject the temporarily grafted marrow, and is designated "grafted-marrow-rejection mortality"

The delayed type of mortality is not associated with secondary hematopoietic depression, is preceded by loss of body weight and atrophy of lymphoid tissue, and occurs only in those combinations of strains in which the donor strain can reject tissue grafts from the recipient strain, regardless of whether the reverse is true A similar fatal disease has been obtained by the transplantation of lymphoid tissue from parent inbred strains into their unirradiated, genetically tolerant $F_1$ hybrid mice This type of mortality is therefore attributed to an immunological reaction of the successfully grafted homologous lymphoid elements or their bone marrow progenitors against the foreign tissue antigens of the tolerant host The terms homologous disease and heterologous disease have been proposed to designate the specific complications and mortality resulting from the successful transplantation of homologous or heterologous, as opposed to isologous or autologous, bone marrow, or lymphoid tissue into a tolerant host, regardless of the reason for tolerance on the part of the host

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*References*

1 SYMPOSIUM ON ANTIBODIES THEIR PRODUCTION AND MECHANISM OF ACTION 1957 J Cellular Comp Physiol **50** (Suppl 1)
2 MAIN, J M & R T PREHN 1955 Successful skin homografts after the administration of high dosage X radiation and homologous bone marrow J Natl Cancer Inst **15.** 1023–1030
3 TRENTIN, J J 1956 Mortality and skin transplantability in X-irradiated mice receiving isologous, homologous or heterologous bone marrow Proc Soc Exptl Biol Med **92:** 688–693
4 BROCADES ZAALBERG, O, O VOS & D W VAN BEKKUM 1957 Surviving rat skin grafts in mice Nature **180** 238–239
5 MAIN, J M & R T PREHN 1957 Fate of skin homografts in X-irradiated mice treated with homologous marrow J Natl Cancer Inst **19.** 1053–1064
6 TRENTIN, J J 1956 Effect of X-ray dose on mortality and skin transplantability in mice receiving $F_1$ hybrid marrow Proc Soc Exptl Biol Med **93.** 98–100
7 SNELL, G D 1953 Transplantable tumors *In* The Physiopathology of Cancer : 338–391 Hoeber-Harper New York, N Y
8 BILLINGHAM, R E, L BRENT & P B MEDAWAR 1956 'Enhancement' in normal homografts, with a note on its possible mechanism Transplantation Bull **3:** 84–88
9 BILLINGHAM, R E, L BRENT & P B MEDAWAR 1956 Quantitative studies on tissue transplantation immunity III Actively acquired tolerance Phil Trans Roy Soc London Ser B **239**· 357–414
10 TRENTIN, J J 1957 The immunological basis for induced tolerance to skin homografts in irradiated mice receiving bone marrow transfusions Transplantation Bull **4** 74–76
11 VAN BEKKUM, D W & O VOS 1957 Immunological aspects of homo- and heterologous bone marrow transplantation in irradiated animals J Cellular Comp Physiol **50** (Suppl 1)· 139–156
12 BARNES, D W H & J F LOUTIT 1954 What is the recovery factor in spleen? Nucleonics **12.** 68–71
13 CONGDON, C C & I URSO 1957 Homologous bone marrow in the treatment of radiation injury in mice Am J Pathol **33.** 749–767
14 NOWELL, P C, L J COLE, J G HABERMEYER & P L ROAN 1956 Growth and continued function of rat marrow cells in X-radiated mice Cancer Research **16.** 258–261
15 GENGOZIAN, N & T MAKINODAN 1957 Mortality of mice as affected by variations

of the x-ray dose and number of nucleated rat bone marrow cells injected Cancer Research 17. 970–975

16 BILLINGHAM, R E, L BRENT & P B MEDAWAR 1954 Quantitative studies on tissue transplantation immunity II The origin, strength and duration of actively and adoptively acquired immunity Proc Roy Soc. London B143: 58–80

17 MITCHISON, N A 1955 Studies on the immunological response to foreign tumor transplants in the mouse I The role of lymph node cells in conferring immunity by adoptive transfer J Exptl Med 102: 157–177

18 GOOD, R. A., R. L VARCO, J B Aust & S J. ZAK 1957 Transplantation studies in patients with agammaglobulinemia Ann N Y Acad Sci 64(5). 882–928

19 TRENTIN, J J 1957 Induced tolerance and "homologous disease" in X-irradiated mice protected with homologous bone marrow Proc Soc Exptl Biol Med 96· 139–144

20 UPHOFF, D E 1957 Genetic factors influencing irradiation protection by bone marrow I The $F_1$ hybrid effect J Natl Cancer Inst 19: 123–130

21 SCHWARTZ, E E, A C UPTON & C C CONGDON 1957 A fatal reaction caused by implantation of adult parental spleen tissue in irradiated $F_1$ mice Proc Soc Exptl Biol Med 96: 797–800

22 FORD, C E, J L HAMERTON, D W H BARNES & J F LOUTIT 1956 Cytological identification of radiation-chimaeras Nature 177: 452–454

23 BARNES, D W H, C E FORD, P L T ILBERY, P C KOLLER & J F LOUTIT 1957 Tissue transplantation in the radiation chimera J Cellular Comp Physiol. 50 (Suppl 1): 123–138

24 MERWIN, R M & C C CONGDON 1957 Repopulation of hematopoietic tissues and blood in lethally X-irradiated mice by homologous bone marrow cells J Natl Cancer Inst 19: 875–884

25 GENGOZIAN, N, I S URSO, C C CONGDON, A D CONGER & T MAKINODAN 1957 Thymus specificity in lethally irradiated mice treated with rat bone marrow Proc Soc Exptl Biol Med 96· 714–720

26 TRENTIN, J J 1957 Thymic weight recovery with homologous and isologous bone marrow in irradiated mice Radiation Research 7: 455

27 MITCHISON, N A 1956 The colonisation of irradiated tissues by transplanted spleen cells Brit J Exptl Pathol 37: 239–247

28 CONGDON, C C, T MAKINODAN & N GENGOZIAN 1957 Effect of injection of rat bone marrow on reticular tissues of mice exposed to X-radiation in the midlethal dose range J Natl Cancer Inst 18 603–614

29 WEYZEN, W W H & O VOS 1957 Production of rat serum proteins in irradiated mice. Nature 180 288–289

30 GRABAR, P, J COURCON, P L T ILBERY, J F LOUTIT & J P MERRILL 1957 Étude immuno-electrophorétique du sérum de souris irradiées par les doses léthales de rayons X et protégées par des cellules de la moelle osseuse de rats Compt rend 245· 950–952

31 SIMONSEN, M 1957 The impact on the developing embryo and newborn animal of adult homologous cells Acta Pathol Microbiol Scand 40: 480–500

32 BILLINGHAM, R E & L BRENT 1957 A simple method for inducing tolerance of skin homografts in mice. Transplantation Bull 4 67–71

# STUDIES OF IMMUNOLOGICAL TOLERANCE TO NERVOUS TISSUE IN RATS*

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Several species of laboratory animals are known to develop experimental encephalomyelitis following injection of adult mammalian nervous tissue emulsified in Freund's complete adjuvant.[1-8] This disease appears approximately two weeks after injection and is characterized clinically by paralysis and, pathologically, by focal areas of vascular and perivascular inflammation confined to the brain, spinal cord, and leptomeninges. Both heterologous and homologous nervous tissues emulsified in complete adjuvant are paralytogenic for susceptible animals. In addition, Kabat *et al.*[9] have found that injection of autologous brain in complete adjuvant induces encephalomyelopathy in monkeys. All available data suggest that an immunological mechanism is responsible for experimental encephalomyelitis.[10] This disease appears to be a useful tool for studying the nature of antibodies against organs implicated in certain forms of tissue injury, including homograft rejection.

The studies of Billingham *et al.*,[11, 12] Billingham and Brent,[14, 15] and other workers[16-18] have shown that immunological tolerance to tissue antigens involved in transplantation immunity may be induced in fetal or newborn animals by injection of homologous cells obtained from adult donor animals. Animals pretreated in this manner later may display, during adult life, little if any immunological response to tissue homografts derived from the donor animals. Homografts remain in place and viable for weeks or months. Other investigators[19-25] have reported induction of immunological unresponsiveness to various heterologous antigens in animals and birds injected with these antigens during fetal or early neonatal life. Reinjection of the same antigen used for pretreatment weeks or months later elicits little or no production of circulating antibodies. These various studies indicate that, under as yet incompletely defined conditions, animals injected with antigenic material during embryonic or early neonatal life may subsequently show impaired immunological response against the same antigenic material during adult life. It is important to point out, however, that some workers[26-28] have reported unsuccessful attempts to induce immunological unresponsiveness in chick embryos and in fetal or newborn rats injected with heterologous antigens.

The present studies were undertaken to determine whether newborn rats injected with guinea pig spinal cord would later show a decreased capacity to develop experimental encephalomyelitis after injection of paralytogenic nervous

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tissue-adjuvant emulsions In addition, the capacity of such animals to produce circulating antibrain antibodies was determined.

## *Materials and Methods*

Twenty-two litters of Wistar rats were pretreated 1, 9, 14, or 28 days after birth as follows Approximately one half of each litter was injected intraperitoneally with a 33 per cent suspension of adult guinea pig spinal cord in physiological saline The other half of each litter was injected intraperitoneally with a 33 per cent suspension of adult guinea pig kidney in saline or was not injected at all The guinea pig spinal cords and kidneys were collected aseptically and were usually stored at −20° C for 1 to 7 days before being homogenized for injection of the rat litters; freshly obtained tissues were used for injection of 3 litters of animals Each rat received 75 to 500 mg of tissue (wet weight), depending on its age and weight at the time of pretreatment The amount of tissue injected per rat was equivalent to 10 to 16 mg of tissue (wet weight) per gram of body weight in 6 of the 7 litters of rats in which each animal was weighed, 1 litter received 4 mg of tissue (wet weight) per gram of body weight Weighed litters included 2 litters of rats pretreated 1 day after birth and all litters pretreated 14 to 28 days after birth

All 22 litters of rats were later injected intracutaneously with 115 mg of guinea pig spinal cord emulsified in adjuvant when they were 8 to 10 weeks old The rats were observed for clinical neurological abnormalities for approximately 3½ weeks They were then bled to obtain sera for complement fixation tests and were sacrificed, and their brains and spinal cords were removed for histological examination Not less than 3 paraffin sections of brain and 4 paraffin sections of spinal cord from each animal were stained with hematoxylin and eosin and then examined microscopically for the presence of lesions indicative of experimental encephalomyelitis

The methods employed for preparing Freund's complete adjuvant and nervous tissue–adjuvant emulsions and the technique used for injection of rats with spinal cord emulsified in adjuvant have been described by Lipton and Freund[7] The complement fixation tests were performed as previously described[6,29] Rat sera were tested in dilutions of 1:4 to 1:1024 against alcoholic extracts of lyophilized rat brain prepared as previously described,[29] the rat brain extracts were diluted 1:250 in saline for use as antigen Sera and antigens gave neither nonspecific reactions nor anticomplementary effects in these dilutions

## *Results*

The occurrence of experimental encephalomyelitis in litters of rats pretreated 1 day after birth and later injected with spinal cord in adjuvant is shown in TABLE 1

As shown in TABLE 1, only 14 of 46 newborn rats pretreated with spinal cord later showed evidence of encephalomyelopathy The disease in these 14 animals tended to be somewhat delayed in onset and mild in character Eight of the 14 rats had clinical signs, these signs were usually mild and, in 3 animals,

TABLE 1
EXPERIMENTAL ENCEPHALOMYELITIS IN RATS PRETREATED ONE DAY AFTER BIRTH AND LATER INJECTED WITH SPINAL CORD IN ADJUVANT

| Pretreatment: Material injected | Pretreatment: Amt injected (mg /rat) | No of rats | Occurrence of encephalomyelitis following injection of spinal cord-adjuvant* | |
|---|---|---|---|---|
| Spinal cord | 75 | 46 | 14/46† | (30%) |
| Kidney | 75 | 28 | 25/28 | (89%) |
| Uninjected | — | 17 | 15/17 | (88%) |

* One hundred and fifteen mg spinal cord in adjuvant at 8 to 10 weeks of age
† Numerator, number of rats with encephalomyelitis, denominator, number of rats injected

did not appear until 20 to 23 days after injection of spinal cord in adjuvant All 14 rats had histological evidence of encephalomyelitis, but the neurological lesions tended to be infrequent and minimal Thirty-two of the 46 newborn rats pretreated with spinal cord had no evidence of damage to the central nervous system

Twenty-five of 28 littermates pretreated with kidney and 15 of 17 littermates left uninjected later showed typical encephalomyelitis The majority of these animals developed severe paralysis of the hind legs 12 to 15 days after injection of spinal cord in adjuvant and had intense disseminated central nervous system lesions

The occurrence of experimental encephalomyelitis in the litters of rats pretreated 9 to 28 days after birth is shown in TABLE 2, which shows that 6 of 13 rats injected with spinal cord 9 days after birth later developed encephalomyelop-

TABLE 2
EXPERIMENTAL ENCEPHALOMYELITIS IN RATS PRETREATED 9, 14, OR 28 DAYS AFTER BIRTH AND LATER INJECTED WITH SPINAL CORD IN ADJUVANT

| Pretreatment: Days after birth | Pretreatment: Material injected | Pretreatment: Amount injected* (mg /rat) | No of rats | Occurrence of encephalomyelitis after injection of spinal cord-adjuvant† | |
|---|---|---|---|---|---|
| 9 | Spinal cord | 100 | 13 | 6/13‡ | (46%) |
| | Kidney | 100 | 10 | 9/10 | (90%) |
| 14 | Spinal cord | 150 | 16 | 13/16 | (81%) |
| | Kidney or uninjected | 150 | 13 | 13/13 | (100%) |
| 28 | Spinal cord | 500 | 12 | 10/12 | (83%) |
| | Kidney or uninjected | 500 | 11 | 10/11 | (91%) |

* Additional experiments in which a few litters of rats were pretreated with the same amount of tissue irrespective of body weight gave even more striking results
† Given 115 mg spinal cord in adjuvant at 8 to 10 weeks of age
‡ Numerator, number of rats with encephalomyelitis, denominator, number of rats injected

athy The disease in these 6 rats was mild Nine of 10 littermates developed typical disease Severe disease occurred in 23 of 28 animals pretreated with spinal cord 14 or 28 days after birth and in 23 of 24 littermate controls

Serologic tests revealed that the 22 litters of rats did not differ materially with respect to production of complement fixing antibrain antibodies Complement fixing antibrain antibody titers of 1:4 or greater occurred in 60 to 82 per cent of those animals pretreated with spinal cord Such titers occurred in 67 to 95 per cent of littermate controls pretreated with kidney or left uninjected Serum samples were obtained from 3 litters of rats pretreated 1 day after birth and from all litters of rats pretreated 9 to 28 days after birth, 1 to 3 days before the injection of spinal cord in adjuvant, all of these sera had antibrain antibody titers of less than 1 4

### *Discussion*

The data of TABLE 1 indicate that newborn rats pretreated with spinal cord may later show a marked decrease in their capacity to develop experimental encephalomyelopathy during adult life The majority of these animals have neither clinical nor histological evidence of encephalomyelopathy after injection of spinal cord emulsified in adjuvant When the disease does occur it tends to be mild Newborn littermate controls pretreated with kidney or not injected at all later develop severe encephalomyelitis after the injection of nervous tissue–adjuvant emulsions Thus, newborn rats injected with spinal cord have a diminished capacity to develop experimental encephalomyelitis after injection of spinal cord–adjuvant at 8 to 10 weeks of age

The data of TABLE 2 suggest that rats pretreated with spinal cord as late as 9 days after birth may subsequently show some reduction in their capacity to develop encephalomyelitis The occurrence of disease observed in these animals was less than that noted in littermates pretreated with kidney Moreover, the disease in rats injected with spinal cord 9 days after birth was mild compared to that observed in the littermate controls Pretreatment of rats with spinal cord 14 or 28 days after birth does not appear to diminish the capacity of these animals subsequently to develop typical encephalomyelitis Although small numbers of animals were employed for these experiments concerning pretreatment at 9 to 28 days of age, the findings are in agreement with the work of Woodruff and Simpson[16] and of Woodruff[17, 30] dealing with tolerance to skin homografts in rats These workers found that homograft tolerance could be induced in rats during the first 1 to 2 weeks of life Thus, rats pretreated with spinal cord 1 or 9 days after birth appear subsequently to exhibit immunological tolerance to the paralytogenic activity of spinal cord–adjuvant emulsions, pretreatment 14 or 28 days after birth appears to induce little if any tolerance to paralytogenic spinal cord–adjuvant emulsions

In contrast, pretreatment of rats at 1, 9, 14, or 28 days after birth does not seem materially to suppress later production of complement fixing antibodies against the antigen or antigens in the alcoholic extracts of rat brain employed for the serologic tests Previous studies[8, 6, 29, 31] have not revealed a direct correlation between development of paralysis and production of

circulating antibrain antibodies The serologic portion of this work provides evidence for the thesis that complement fixing antibodies against alcoholic extracts of brain do not play a primary role in initiating the tissue injury characterizing experimental encephalomyelitis The significance of this observation is limited by the fact that the antigenic material has not been defined

A tentative interpretation of the biological significance of these studies is possible in view of what is known about the delayed appearance of paralytogenic activity in nervous tissue of certain species of animals and in the light of the "self-marker" theory proposed by Burnet and Fenner [32, 33] Other workers[2, 34, 35] have found fetal human and fetal rabbit brain to be nonparalytogenic, paralytogenic activity was present in 3-day-old rabbit spinal cord and 12-day-old rabbit brain Work in our laboratory indicates that the brain of the newborn rat has no demonstrable paralytogenic activity for adult rats and guinea pigs, whereas adult rat brain is paralytogenic for both of these species of animals [36] According to Burnet and Fenner's "self-marker" theory, if certain constituents do not appear in living nervous tissue until after birth, they would be recognized as "nonself" rather than "self" These constituents would be potentially antigenic and paralytogenic This concept would account for the finding of Kabat *et al* [9] that autologous brain induces encephalomyelopathy in monkeys It is conceivable that paralytogenic constituents of adult spinal cord injected into neonatal rats are treated as "self" Such rats would subsequently recognize paralytogenic material reinjected during later life as "self" These animals would exhibit a diminished capacity to develop experimental encephalomyelitis after injection of paralytogenic nervous tissue–adjuvant emulsions

## *Conclusions*

(1) Newborn rats pretreated with spinal cord and later tested when 8 to 10 weeks old appear to display immunological tolerance to the paralytogenic activity of nervous tissue–adjuvant emulsions

(2) Rats pretreated with spinal cord as late as 9 days after birth later appear to show some tolerance to the paralytogenic activity of nervous tissue, pretreatment with spinal cord at 14 or 28 days after birth appears to induce little, if any, tolerance

(3) Pretreatment of neonatal rats with spinal cord does not appear to alter materially the capacity of these animals later to produce complement fixing antibrain antibodies against alcoholic extracts of rat brain

## *Acknowledgments*

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## *References*

1 Morgan, I M 1947 Allergic encephalomyelitis in monkeys in response to injection of normal monkey nervous tissue J Exptl Med 85 131

2 Kabat, E A, A. Wolf & A E Bezer 1947 The rapid production of acute disseminated encephalomyelitis in rhesus monkeys by injection of heterologous and homologous brain tissue with adjuvants J Exptl Med 85: 117

3 FREUND, J, L R STERN & T M PISANI 1947 Isoallergic encephalomyelitis and radiculitis in guinea pigs after one injection of brain and mycobacteria in water-in-oil emulsion J Immunol 57: 179
4 MORRISON, L R 1947 Disseminated encephalomyelitis experimentally produced by the use of homologous antigen Arch Neurol Psychol 58: 391
5 OLITSKY, P K & R H YAGER 1949 Experimental disseminated encephalomyelitis in white mice J Exptl Med 90 213
6 THOMAS, L, P. Y PATERSON & B SMITHWICK 1950 Acute disseminated encephalomyelitis following immunization with homologous brain extracts I Studies on the role of a circulating antibody in the production of the condition in dogs. J Exptl Med 92. 133
7 LIPTON, M M & J FREUND 1953 Allergic encephalomyelitis in the rat induced by the intracutaneous injection of central nervous system tissue and adjuvants J Immunol 71: 98
8 PATERSON, P Y & E D BRAND 1957 Production of experimental encephalomyelitis in a new host, the cat Federation Proc 16 428
9 KABAT, E A, A WOLF & A E BEZER 1949 Studies on acute disseminated encephalomyelitis produced experimentally in rhesus monkeys IV Disseminated encephalomyelitis produced in monkeys with their own brain tissue J Exptl Med 89: 395
10 PATERSON, P Y 1958 Organ-specific tissue damage induced by mammalian tissue-adjuvant emulsions *In* Cellular and Humoral Aspects of the Hypersensitive States H S Lawrence, Ed, Hoeber New York, N Y In press
11 BILLINGHAM, R E, L BRENT & P B MEDAWAR 1953 Actively acquired tolerance of foreign cells Nature 172: 603
12 BILLINGHAM, R E, L BRENT & P B MEDAWAR 1956 Quantitative studies on tissue transplantation immunity III Actively acquired tolerance. Phil Trans Roy Soc London B239 357
13 MEDAWAR, P B 1956 Introductory remarks Proc Roy Soc B146 1
14 BILLINGHAM, R E & L BRENT 1956 Acquired tolerance of foreign cells in newborn animals Proc Roy Soc B146: 78
15 BILLINGHAM, R E & L BRENT 1957 A simple method for inducing tolerance of skin homografts in mice Transplantation Bull 4 67
16 WOODRUFF, M F A & L O SIMPSON 1955 Induction of tolerance to skin homografts in rats by injection of cells from the prospective donor soon after birth Brit J Exptl Pathol 36: 494
17 WOODRUFF, M F A. 1957 Postpartum induction of tolerance to homologous skin in rats Ann N Y Acad Sci 64(5): 792
18 MARTINEZ, C, J B AUST & R A GOOD 1956 Acquired tolerance to ovarian homografts in castrated mice Transplantation Bull 3. 128
19 HANAN, R & J OYAMA 1954 Inhibition of antibody formation in mature rabbits by contact with the antigen at an early age J Immunol 73: 49
20 BUXTON, A 1954 Antibody production in avian embryos and young chicks J Gen Microbiol 10 398
21 DIXON, F J & P H MAURER 1955 Immunologic unresponsiveness induced by protein antigens J Exptl Med 101 245
22 CINADER, B & J M DUBERT 1955 Acquired immune tolerance to human albumin and the response to subsequent injections of diazo human albumin Brit J Exptl. Pathol 36 515
23 SMITH, R T & R A BRIDGES 1956 Response of rabbits to defined antigens following neonatal injection Transplantation Bull 3 145
24 SIMONSEN, M 1956 Actively acquired tolerance to heterologous antigens Immunological consequences of transfusing chicken embryos with human blood Acta Pathol Microbiol Scand 39 21
25 WOLFE, H R, C TEMPELIS, A MUELLER & S REIBEL 1957 Precipitin production in chickens XVII The effect of massive injections of bovine serum albumin at hatching on subsequent antibody production J Immunol 79 147
26 BURNET, F M, J D STONE & M EDNEY 1950 The failure of antibody production in the chick embryo Australian J Exptl Biol Med Sci 28: 291
27 BAUER, J A, P E PECKHAM & A G OSLER 1956 Effect of fetal or neonatal immunization on antibody response of the adult rat Proc Soc Exptl Biol Med 92 714
28 COHN, M 1957 The problem of specific inhibition of antibody synthesis in adult animals by immunization of embryos Ann N Y Acad Sci 64(5). 859
29 PATERSON, P Y 1957 A study of experimental encephalomyelitis employing mammalian and nonmammalian nervous tissues J Immunol 78: 472

30 Woodruff, M F A. 1957 Further observations on induction of tolerance of skin homografts in rats Transplantation Bull 4 157
31 Lumsden, C E, E A Kabat, A Wolf & A E Bezer 1950 Studies on acute disseminated encephalomyelitis produced experimentally in rhesus monkeys V Complement-fixing antibodies J Exptl Med **92:** 253
32 Burnet, F M & F Fenner 1949 The Production of Antibodies 2nd ed Macmillan Melbourne, Australia
33 Burnet, F M 1956 Enzyme, Antigen and Virus Cambridge Univ Press London, England
34 Alvord, E C, Jr 1949 Studies on the etiology and pathogenesis of experimental meningoencephalomyelitis in the guinea pig J Immunol **61:** 355
35 Kabat, E A, A Wolf & A E Bezer 1948 Studies on acute disseminated encephalomyelitis produced experimentally in rhesus monkeys III J Exptl Med **88** 417
36 Paterson, P Y (Unpublished data)

## *Discussion of the Paper*

Byron H Waksman (*Massachusetts General Hospital, Boston, Mass*) Paterson's data support the attractive hypothesis that an antigen absent from the organism during the "tolerance period" of development fails to induce tolerance and is therefore regarded in later life as a foreign substance and can act as an autoantigen In rats, central nervous system myelin begins to be laid down in the first 2 or 3 days following birth, but the encephalitogenic antigen remains low in concentration for at least 1 or 2 weeks Since the tolerance period ends within the first few days after birth, this theory is tenable with regard to myelin as an autoantigen, obviously it may also explain the autoantigenicity of peripheral nerve myelin and of the spermatocyte However, autoallergies comparable in all histological and other details to experimental allergic encephalitis, neuritis, and orchitis affect organs that are present in essentially their mature form before birth, among them the crystalline lens, the uvea, and the thyroid It would seem that a more generally applicable hypothesis might take into account, not only the simple presence or absence of a given antigen during the tolerance period, but also the presence of barriers separating the antigenic tissue from the circulation and, indeed, the possibility that intact antigen may be released in negligible amounts into the circulation in the course of normal metabolism

That these other considerations may play a role in determining an animal's ability to react to a particular autoantigen as a foreign substance even in adult life is indicated by a number of observations Ferraro and his co-workers[1] found that injections of nervous tissue in adult guinea pigs would diminish their later ability to develop allergic encephalomyelitis when injected with nervous tissue plus the Freund adjuvant Condie *et al*[2] produced an almost complete inhibition of the ability of the adult rabbit to develop allergic encephalomyelitis by a preliminary course of subcutaneous injections of homologous spinal cord We have obtained similar results in the rat,[3] weekly subcutaneous injections of rat spinal cord begun at the age of 3 weeks produced a marked decrease in the disease which could be elicited later It is of considerable interest to note that, in the experiments of the Minnesota group,[2] a single injection in the adult rabbit was almost ineffectual, the total dose being the same Similarly, Paterson has shown that a single injection in the rat at 14 or 28 days had essentially no effect, although the injected dose was larger than the

total in our rats, which were injected repeatedly Is it possible that the single injection at birth produces the same effect as repeated injections later, the difference perhaps being due to inadequacy of catabolic mechanisms in the newborn?

We are too greatly preoccupied with the word tolerance I echo the plea made by Kabat in the monograph preceding this[4] that we keep clearly in mind the different types of experiment in which specific immunological nonreactivity has thus far been demonstrated experimentally Tolerance is a word proposed by Billingham and his colleagues[5] to describe the nonreactivity present in animals that receive antigen during a restricted period of early development, the antigen being most often present in living cells that persist, and presumably multiply, in the treated animal, as Billingham has shown elsewhere in this monograph "Paralysis" describes the state produced in adult animals by overwhelming doses of an antigen, especially if it is a substance catabolized by the individual with difficulty, if at all [6] The term unresponsiveness was used by Chase and Battisto[7, 8] to designate the condition of animals fed small-molecular allergens for a protracted period and that were subsequently unable to react to these with delayed (contact) sensitization or, to a lesser degree, with antibody formation Finally, I call attention to the fact discovered by Swift and his colleagues in studies of streptococci[9] and by Julianelle in studies of pneumococci[10] that rabbits injected intradermally with antigen develop delayed sensitivity, while those injected subcutaneously or intravenously, especially in large or repeated doses, form antibodies, but are at the same time rendered incapable of developing delayed sensitivity The inhibition of allergic encephalomyelitis described by Ferraro *et al*,[1] by Condie *et al*,[2] and in this monograph by Paterson, as well as in our own experiments resembles, if anything, this last phenomenon or, perhaps Battisto and Chase's unresponsiveness,[8] since antibody formation is not affected, since it may be produced in adult animals and since, in these cases, repetition is a necessary feature of the experiment, the injections being subcutaneous or intraperitoneal

## *References*


1 FERRARO, A, L ROIZIN & C L CAZZULLO 1950 Experimental studies in allergic encephalomyelitis Prevention and production Note III J Neuropathol 9. 18–28

2 CONDIE, R M, J T KELLY, B CAMPBELL & R A GOOD 1957 Prevention of experimental allergic encephalomyelitis by prior injections of homologous spinal cord (Abstr ) Federation Proc 16 24

3 WAKSMAN, B H The effect of pre-treatment with nervous tissue on the production of allergic encephalomyelitis in experimental animals In preparation

4 KABAT, 1957 Discussion of R A. Good *et al* Ann N Y Acad Sci 64·(5) 924–926

5 BILLINGHAM, R E, L BRENT & P B MEDAWAR 1953 Actively acquired tolerance of foreign cells Nature 172· 603–606

6 FELTON, L D, G KAUFFMAN, B PRESCOTT & B OTTINGER 1955 Studies on the mechanism of the immunological paralysis induced in mice by pneumococcal polysaccharides J Immunol 74 17–26

7 BATTISTO, J R & M W CHASE 1955 Immunologic paralysis in guinea pigs fed allergenic chemicals (Abstr ) Federation Proc 14. 456–457

8 BATTISTO, J R & M W CHASE 1955 Further studies on the state of inhibition to drug sensitization induced by feeding of the drug (Abstr ) Bacteriol Proc 55 94–95

9 DERICK, C L & H F SWIFT 1929 Reactions of rabbits to nonhemolytic streptococci J Exptl Med 49 615–636, 883–897

10 JULIANELLE, L A 1930 Reactions of rabbits to intracutaneous injections of pneumococci and their products J Exptl Med 51· 441–472, 625–658

# STUDIES ON BONE MARROW HOMOTRANSPLANTATION IN X-IRRADIATED RABBITS*

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## *Introduction*

During the past decade most of the research on postirradiation bone marrow transplantation has been conducted on mice and rats It has been shown that these rodents can recover from an otherwise lethal dose of whole-body X irradiation if they are treated by intravenous injections of bone marrow This beneficial effect is now known to be due to the repopulation of hemopoietic tissues in the depleted host by the injected cells and their descendants,[1] leading to rapid hematological recovery and prevention of death from infection or hemorrhage In the only reported work on rabbits during this period[2] there was failure to achieve successful marrow grafts

This paper is a brief account of our recent studies on bone marrow transplantation in rabbits, using the sex difference in the granulocytes as a means of identifying the homologous cells and their descendants after injection By transfusing marrow from a female animal into an irradiated male, survival and proliferation of the donor cells in the host have been deduced if typical female cells can later be identified in the host's blood and marrow

## *Materials and General Methods*

Young adult male New Zealand white rabbits, not inbred in the genetic sense, weighing 2 5 to 3 kg were exposed to whole-body X irradiation The X rays were administered from a Phillips machine operating at 250 kVp, 15 mAmp , 50 cm target distance, 0 4 mm Sn, 0 25 mm Cu, and 1 0 mm Al filters, HVL 2 5 mm Cu, 50 r/min dose rate † Within 1 to 3 hours after the final irradiation each animal was given an intravenous injection of a saline suspension of 1 2 billion nucleated marrow cells from a female donor Total leukocyte counts, hemoglobins, and microhematocrits were determined on the irradiated animals every 2 days for the first 30 days and once per week thereafter Blood films were taken from the ear at the same time intervals, stained by Wright's method, and the polymorphonuclear leukocytes examined Female leukocytes are characterized by a nuclear appendage called a "drumstick" by Davidson and Smith,[3] and consisting of a well-defined chromatin nodule about

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† We are indebted to G D'Angio and to the X-Ray Department of the Children's Hospital, Boston, Mass , for granting us radiation facilities

1.5 μ in diameter, joined by a single fine chromatin strand to one lobe of the nucleus These drumsticks can be seen in approximately 7 per cent of the granulocytes in blood films from female rabbits[4] Before accepting the fact that the leukocytes were derived at least in part from the transplanted female bone marrow it was arbitrarily decided that a minimum of 6 cells showing typical drumsticks should be found.

All animals that died were autopsied, and marrow from the midpoint of the shaft of the right femur and tissue from the thymus, spleen, mesenteric lymph nodes, and appendix were taken, fixed in Helly's fluid, and stained with hematoxylin and eosin

*Experiments and Results*

*Demonstration of marrow transplantation* Experiments with homologous bone marrow transplantation after the recipient animal had been exposed to varying doses of whole-body X irradiation[4] showed that initially successful marrow transplants occurred in 15 per cent of the animals receiving 800 r ($LD_{60}$ 30 days), 38 per cent of those receiving 900 r, and 62 per cent of those exposed to 1000 r Success was indicated by rapid restoration of the leukocyte count to normal and the appearance of heterophils bearing nuclear chromatin drumsticks characteristic of the female Microscopic studies confirmed the bone marrow repopulation and showed rapid recovery of lymphoid tissue in the treated animals

*Demonstration of transplantation of marrow preserved in glycerol at −70° C* Successful marrow homotransplants in rabbits exposed to 900 r whole-body X irradiation were shown to occur about as frequently following the intravenous injection of preserved marrow as following the administration of fresh marrow[5] This preservation was done by the Polge-Smith-Parkes method using, with a few minor modifications, the small freezing apparatus described and illustrated in the paper by Barnes and Loutit[6] The marrow, in sealed tubes containing 15 per cent glycerol in the serum of the donor, was cooled at approximately 1° C per minute down to −15° C, then, at no more than 10° per minute, to −70° C The tubes were stored for one week in solid $CO_2$ and alcohol When needed, the marrow was rapidly thawed by plunging the tubes into a water bath at 38° C

*Demonstration of hemopoietic transplantation after treatment with neonatal rabbit liver* With the general acceptance of cellular repopulation as the main mechanism by which hemopoietic suspensions bring about their therapeutic effect, it was considered worth while to reinvestigate the reported beneficial effects on X-irradiated rabbits of liver cell suspensions from newborn rabbits[7] and mice[8] Experiments on these lines with rabbits exposed to 900 r showed that blood-forming cells in the rabbit liver suspension survive and repopulate the host's depleted hemopoietic tissues Successful transplantation, however, does not occur following treatment by mouse liver

*Attempted analysis of the late mortality following homologous marrow transplantation* It has been observed in mice that, whereas the life span of irradiated animals treated with isologous marrow is almost normal, many animals treated with homologous or heterologous marrow die between 30 to 80 days after

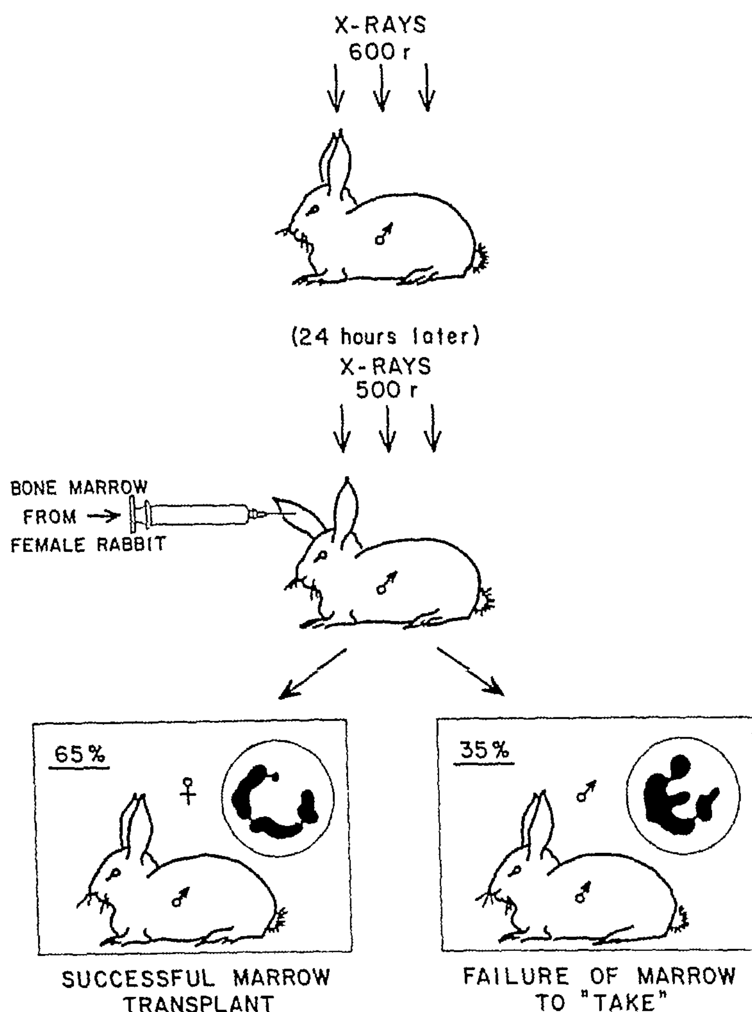



FIGURE 1

irradiation, with a syndrome of diarrhea, wasting, and lymphoid atrophy. The cause of these late deaths is not certain, and this hazard constitutes a serious drawback to any possible application of marrow transplantation to human patients It has been suggested that the responsible mechanism might be a delayed immune reaction on the part of the host, causing destruction of the marrow graft [9]

To test this hypothesis long-term studies of rabbits with bone marrow homotransplants were undertaken [10] As our earlier work had shown that in the rabbit, after an X-ray dosage that permits a large percentage of successful marrow homotransplants (900 to 1000 r), a high mortality occurs initially from shock and, later, from peptic ulceration and perforation, divided doses of irradiation and postirradiation antibiotic therapy were used to minimize these complications Each animal was exposed to 600 r total-body X irradiation, followed 24 hours later by 500 r Immediately after the final dose of X rays they were also given 70 to 75 mg per day of tetracycline hydrochloride* for 3 weeks

Following bone marrow treatment, 65 per cent of the irradiated male animals

* Achromycin, soluble in water, was furnished by Lederle Laboratories Division, American Cyanamid Company, Pearl River, N Y

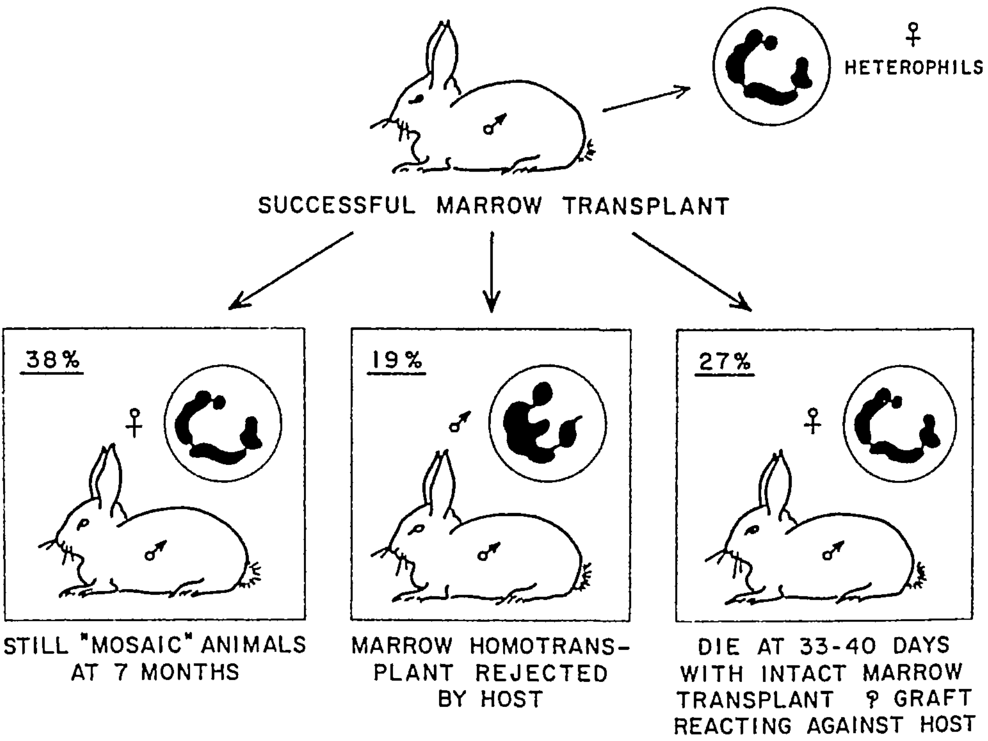



FIGURE 2

showed successful marrow transplants as indicated by rapid restoration of the leukocyte count to normal and the appearance of female cells in the peripheral blood (FIGURE 1) Of these, 16 per cent subsequently died as the result of perforation of a peptic ulcer, in 19 per cent there was rejection of the marrow transplant by the host, 27 per cent died 33 to 40 days after progressive wasting, diarrhea, and leukocytosis, with generalized lymphoid atrophy, but with an intact and functioning marrow transplant, 38 per cent remained alive and well for 7 months with the marrow transplant intact and still contributing female cells to the peripheral blood (FIGURE 2) Of the animals with unsuccessful transplants, some regenerated their own bone marrow and lived Thus, although 40 per cent of the animals given bone marrow survived the 7-month experimental period, not all of these had persistent marrow transplants

## *Discussion*

The data obtained in these experiments add to the considerable evidence already accumulated to show that, in irradiated animals treated with bone marrow, the introduced cells survive and repopulate the depleted hemopoietic tissues of the recipients Freeze preservation of the marrow by the Polge-Smith-Parkes technique causes no appreciable loss of this power The beneficial effect of postirradiation neonatal liver treatment is similarly shown to be due to the presence of primitive blood-forming cells in the liver suspension that persist in the host and lead to rapid hematological recovery There now seems little reason to believe that a humoral factor is of importance following the use of any of these suspensions

Also, further light is shed on the late mortality that occurs in irradiated animals treated with homologous marrow It is evident that, under the conditions of our study, although some delayed deaths were undoubtedly due to rejection of the transplant by the regenerated antibody-producing system of the host, many deaths were due to some other cause

In this latter group (the 27 per cent that died at 33 to 40 days) the syndrome of wasting and diarrhea appears similar to that seen by Congdon and Urso in lethally irradiated mice treated with homologous marrow [11] The results we have just shown exclude destruction of the marrow transplant by a delayed immunological response of the host to the foreign cells as the mechanism in this group of late deaths An alternative hypothesis that the graft is forming antibodies against the host[12] certainly cannot be excluded on this evidence and is at present the most favored possibility

The great advantage of the nuclear "marker" technique used in this work is its simplicity Other labeling methods for use in homologous (as distinct from heterologous) marrow transplantation studies involve either a supply of animals of specific antigenic type[13] or of animals with some distinctive chromosome pattern as in the T6 strain of mice used by Ford *et al* at Harwell, England [1]

As it is probable that a similar morphologic sex difference will be shown to be present in the neutrophils of many mammalian species other than humans,[3] dogs,[14] and rabbits,[15] the method described may prove useful in similar work with other animals and in any clinical studies that eventually may be attempted.

## *References*


1 Ford, C E, J L Hamerton, D W H Barnes & J F Loutit 1956 Cytological identification of radiation-chimeras Nature **177**. 452–454

2 Hilfinger, M F, J H Ferguson & P A. Riemenschneider 1953 The effect of homologous bone marrow emulsion on rabbits after total body irradiation J Lab Clin Med **42**· 581–591

3 Davidson, W H & D R Smith 1954 A morphological sex difference in the polymorphonuclear neutrophil leucocytes Brit Med J **2**: 6–7

4 Porter, K A 1957 Effect of homologous bone marrow injections in X-irradiated rabbits Brit J Exptl Pathol **38** 401–412

5 Porter, K A & J E Murray 1958 Successful homotransplantation of rabbit bone marrow after preservation in glycerol at −70° C Cancer Research **18**. 117–119

6 Barnes, D W H & J F Loutit 1955 The radiation recovery factor preservation by the Polge-Smith-Parkes technique J Natl Cancer Inst **15**· 901–905

7 Jacobson, L O, E K Marks & E O Gaston 1956 The effect of rabbit liver cell suspensions on the survival of irradiated rabbits (Abstr) Radiation Research **5**. 483

8 Jacobson, L O, E K Marks & E O Gaston 1956 Modification of radiation injury in the rabbit Proc Soc Exptl Biol Med **91**: 135–139

9 Cole, L J, J G Habermeyer & V P Bond 1955 Recovery from acute radiation injury in mice following administration of rat bone marrow J Natl Cancer Inst **16**: 1–9

10 Porter, K A & J E Murray 1958 Long-term study of X-irradiated rabbits with bone marrow homotransplants J Natl Cancer Inst **20**: 189–205

11 Congdon, C C & I Urso 1956 Isologous versus homologous bone marrow in the treatment of irradiated mice (Abstr) Radiation Research **5**. 474

12 Trentin, J J 1956 Mortality and skin transplantability in X-irradiated mice receiving isologous, homologous or heterologous bone marrow Proc Soc Exptl Biol Med **92**: 688–693

13 LINDSLEY, D L, T T Odell & F G TAUSCHE 1955 Implantation of functional erythropoietic elements following total-body irradiation. Proc Soc. Exptl Biol Med **90** 512–515
14 PORTER, K A 1957 A sex difference in morphology of neutrophils in dog Nature. **179:** 784–785
15 LUERS, T 1956 Vergleichende Untersuchungen über morphologische Geschlechtsunterschiede der neutrophilen Leukozytenkerne bei Mensch und Kaninchen Blut **2** 81–88

# THE HOMOGRAFT REACTION IN X-IRRADIATED RABBITS TREATED WITH HOMOLOGOUS BONE MARROW A PRELIMINARY REPORT*

*By* Fred Lee, James B Dealy, Jr , Gustave J Dammin, and Marcus S Brooke

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The etiology and pathogenesis of deferred mortality in animals treated with homologous bone marrow after lethal external irradiation has been the subject of wide interest and speculation, largely on the basis of work in the rodent [1-4] In his extension of the marrow graft technique to rabbits that are not inbred in the genetic sense, Porter[5, 6] established the leukocyte sex cell marker as a biological yardstick of marrow graft "take" Using Porter's methods,[7]

TABLE 1

| | Periodic mortality (days) | | | Survival beyond 50 days | Totals |
|---|---|---|---|---|---|
| | 1 to 14 | 15 to 27 | 28 to 50 | | |
| Controls (X ray + saline) | 18 | 0 | 0 | 2 | 20 |
| Immediate X-ray death | 5 | 0 | 0 | 0 | 5 |
| Unsuccessful marrow transplants | 1 | 2 | 1 | 2 | 6 |
| Successful marrow transplants | 0 | 10 | 6 | 19 | 35 |
| | | | | | 66 |

TABLE 2

| Series number of animal | Life span in days | Hematocrit: Average up to 1 week before death (percentages) | Hematocrit: One week prior to death (percentages) | Hematocrit: Terminal (percentages) | White blood count: Average up to 1 week before death | White blood count: Terminal | Serum bilirubin (Mg %) Terminal value (maximum): Direct | Serum bilirubin (Mg %) Terminal value (maximum): Indirect | Indirect Coombs test |
|---|---|---|---|---|---|---|---|---|---|
| | | 36 to 42%* | | | 4–10,000* | | 0 2* | 0 6* | |
| ER 7 | 50 | 33 | 29 | 17 | 7–10,000 | 8,600 | Frankly icteric serum | | + (Weakly) |
| ER 9 | 39 | 38 | 38 | 30 5 | 4–6,000 | 3,050 | 0 1 | 0 2 | Neg |
| ER 16 | 43 | 33 | 32 | 27 | 7–15,000 | 17,000 | 0 7 | 1 6 | + (Weakly) |
| ER 29 | 35 | 38 | 28 | 23 | 3–5,000 | 3,300 | 0 1 | 0 3 | Neg |
| ER 39 | 28 | 38 | 38 | 12 | 10–20,000 | 15,000 | 0 1 | 1 2 | + (Weakly) |
| ER 44 | 29 | 39 | 40 | 29 | 4,800–5,600 | 7,500 | 0 2 | 0 6 | + (Weakly) |

* Normal value

* The work reported in this paper was supported in part by Grants H-1771 and H-2791 from the National Heart Institute, Public Health Service, Bethesda, Md

rabbits that demonstrated marrow homograft survival could be divided roughly into three groups according to (1) duration of survival, (2) clinical features, and (3) morphologic alterations in the tissues Preliminary observations on the histological differences between the so-called late-death animals and the longer-surviving radiation chimeras form the basis of this report Experimental controls for the study comprised 20 animals that received saline infusions following irradiation Eighteen of the rabbits died between the first and fourteenth days, the remaining two survived TABLE 1

Forty-six male rabbits were exposed to 1100 r of total-body irradiation divided into successive daily doses of 600 r and 500 r each[7] There were 5 immediate irradiation deaths, and 6 rabbits showed no evidence of successful marrow homograft Of the latter 6, 4 succumbed between the fifth and the twenty-ninth day, 2 animals remain alive at this writing

Among 35 animals successfully grafted and maintained on antibiotics throughout the duration of the experiment, 3 groups emerged on the basis of



FIGURE 1 Spleen of rabbit ER 29 (shorter-survival group) Two splenic follicles are present (see arrows) that are almost devoid of cells, consisting essentially of condensed stroma The sinusoids of the red pulp are dilated and contain numerous macrophages, with abundant hemosiderin Note that FIGURE 5 (rabbit ER 46), at the same magnification, contains only a single large cellular lymphoid follicle Hematoxylin and eosin ×110

clinical behavior The first (early-death) group of 10 animals never recovered from the initial radiation assault and died within 15 to 27 days A second group of 6 animals showed clinical recovery, including an initial gain in body weight but, despite maintenance of appetite, showed a subsequent decline in weight until their deaths within 28 to 50 days, with a mean survival of 37 days A third group of 19 animals survived beyond 50 days, with occasional deaths thereafter due to obvious infection Thus far there have been no graft rejections in this third group

All 6 animals in the second group, heretofore regarded as late-death[7] animals, showed varying degrees of terminal anemia and, in 3 of them, the terminal sera were frankly icteric The clinical cause of death in 2 animals could have been ascribed to pneumonitis, in the remaining 4 it was not apparent Pertinent clinical and laboratory observations are recorded in TABLE 2

The peripheral blood smears in all animals in this group showed evidence of red cell regeneration, with polychromatophilia reaching its peak at the time of death Indirect Coombs tests, utilizing the recipient's original (pre-X-ray,

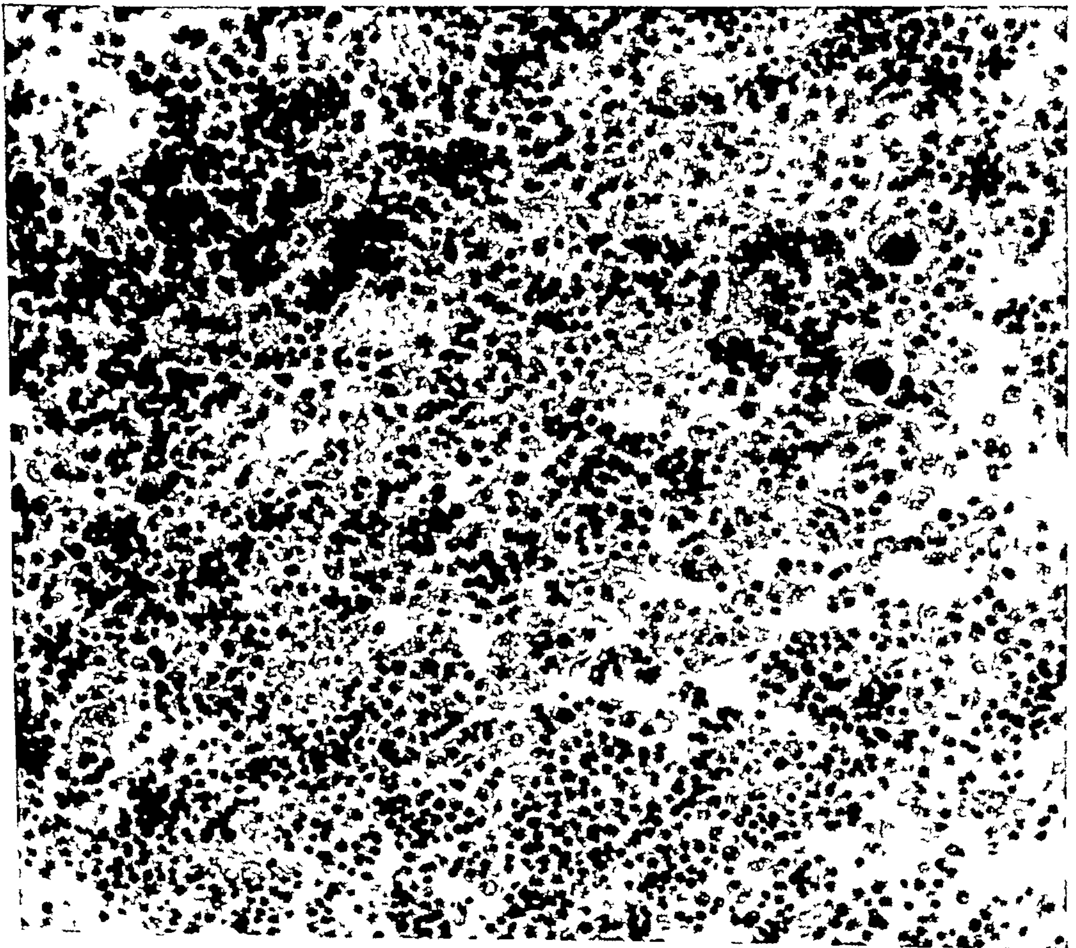


FIGURE 2 Marrow of rabbit ER 16 (shorter-survival group) The marrow is cellular, but many of the cells contain pyknotic nuclei with abnormal chromatin patterns There are few foci of leukocytopoiesis There is serious atrophy of fat, with no normal fat cells present, and congestion is marked Hematoxylin and eosin ×225

premarrow transfusion) red blood cells, were weakly positive in 4 animals, 2 of which (ER 16 and ER 39) also showed definitely elevated indirect serum bilirubin levels in association with a rapid terminal decline in the hematocrit. One of the other 2 animals with a positive indirect Coombs test showed an extremely low hematocrit (ER 7) and had frankly icteric serum. At death, this animal had extensive liver necrosis, but this was unassociated with any inflammatory response, and an almost comparable degree of liver necrosis in animal ER 29 did not correlate with hyperbilirubinemia at any time during life. Direct Coombs tests in animals ER 29 and ER 39 and ER 41 were negative, but these were done only terminally. The circulating white cell counts were generally higher in the animals with the most numerous hemolytic features. The elevated serum bilirubin levels, when present, appeared within 48 hours of death.

Autopsy examinations in this late-death group revealed uniform lymphoid atrophy. The spleens were small, fibrotic, and hypocellular, save for numerous plasma cells as the conspicuous remaining cell type (FIGURE 1). Hemosiderin,



FIGURE 3. Thymus of rabbit ER 16 (shorter-survival group). The thymus contains few cells and is moderately congested. The marked atrophy is characterized by a condensation of the stroma. Hematoxylin and eosin. ×110.



FIGURE 4 Appendix of rabbit ER 44 (shorter-survival group) Lymphocytes are relatively few in the lamina propria and submucosa The mucosa is normal Hematoxylin and eosin ×70

both intracellular and extracellular, was abundant, although there was no good correlation with hematocrit or bilirubin levels While moderately cellular, the bone marrow was distinctly abnormal, with a paucity of cells in the granulocyte series, numerous cells with abnormally distributed nuclear chromatin, and extensive zones of serous fat (FIGURE 2) Sections of the thymus showed advanced atrophy of lymphoid tissue and quantities of serous fat (FIGURE 3) Lymphoid tissue in the appendix was also markedly atrophic (FIGURE 4)

These 6 animals may be compared with 8 of the 19 successfully grafted rabbits that survived more than 50 days Three of these died of pneumonia and 5 were sacrificed At no time after recovery from the initial radiation insult did any of these 8 animals show a decline in weight, a fall in hematocrit or hyperbilirubinemia Indirect Coombs tests carried out in 5 animals were negative

At death, the spleens of the 8 animals that survived more than 50 days were slightly smaller than normal but were 2 to 3 times the size of those in the late-death group (28 to 50 days) Microscopically, splenic architecture was generally preserved, with only moderate congestion (FIGURE 5) Lymphoid follicles were present, many of them of normal size and cell content, with plasma



FIGURE 5 Spleen of rabbit ER 46 (longer-survival group) A large lymphoid follicle containing abundant lymphocytes, as well as a mantle zone, occupies most of the field Compare with FIGURE 1 Hematoxylin and eosin ×110

cells scattered at the periphery Hemosiderin-laden macrophages were few in number The bone marrow (FIGURE 6) and the lymphoid tissue in the thymus gland (FIGURE 7), mesenteric lymph nodes, and vermiform appendices (FIGURE 8) showed cell population and distribution approaching the normal

The foregoing data indicate fairly definite histological differences between the group of rabbits that die with a rather characteristic clinical syndrome from 4 to 7 weeks after successful marrow homograft and those that survive this potentially critical period The positive indirect Coombs tests suggest antibody formation by the radiation chimera, with the inference that it originates in the grafted marrow, since the reaction is upon the recipient's red cells This capacity of the radiation chimera to form antibodies was tested by injecting 100 mg of bovine gamma-globulin intravenously between the tenth and fourteenth days after marrow transfusion, when the peripheral leukocyte count had returned to normal Of the 25 animals in the late-death and chimera groups, only one (ER 16, a late-death animal) has developed a precipitin titer

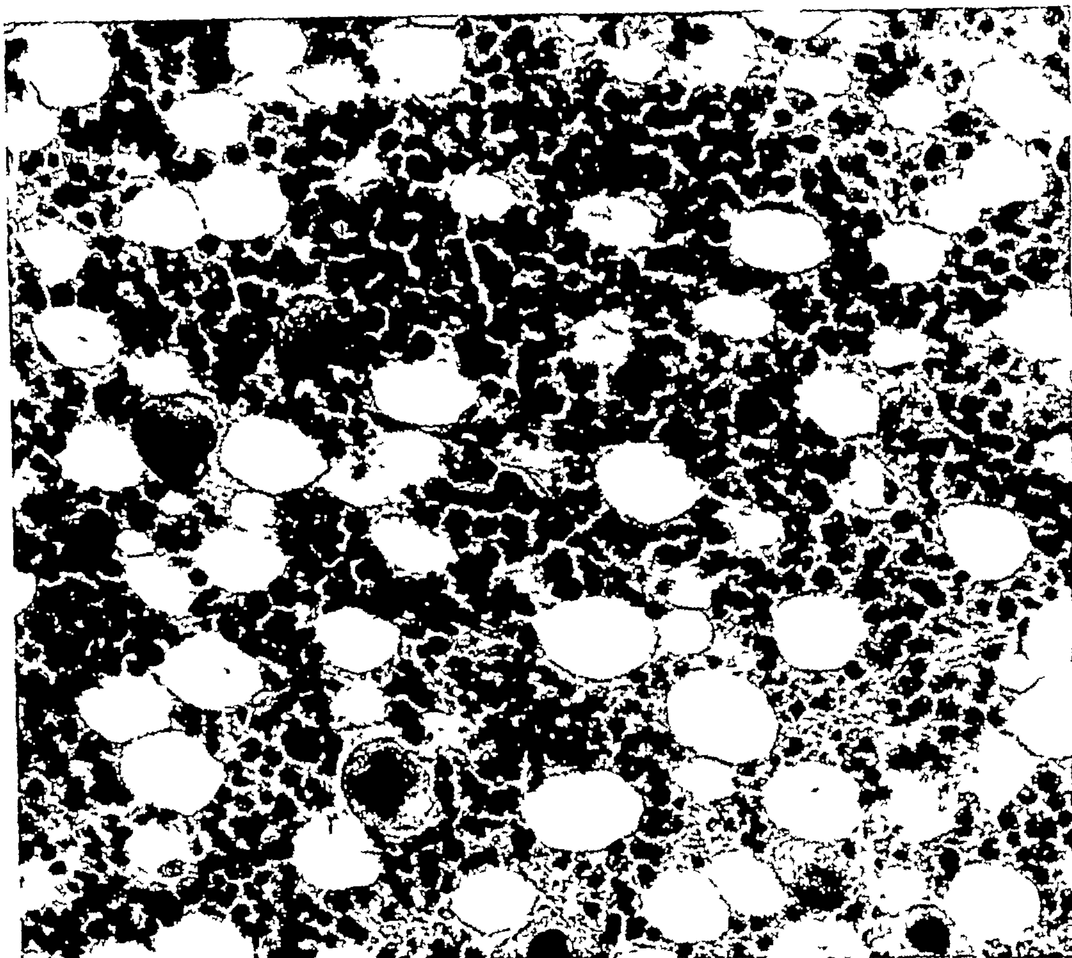


FIGURE 6 Marrow of rabbit ER 42 (longer-survival group) The distribution of cell types in the marrow is essentially normal The fat cells are normal, and there is minimal congestion Compare with FIGURE 2 Hematoxylin and eosin ×310

For reasons not defined by this study, it would appear that the crisis of late death may be attended, in certain instances, by an acute destruction of red cells Work in progress, utilizing chromium- and phosphorus-tagged red cells and $I^{131}$-labeled bovine gamma-globulin disappearance curves following sensitization of either donor or recipient to illuminate further these mechanisms, will be the subject of subsequent reports

The translation of additional histological, hematological, and immunological data to an increasingly accurate clinical identification of the incipient homograft reaction may enable the treatment of it by further irradiation exposure or other means

## *Summary*

A preliminary comparison of the histological features in successfully homografted (marrow) rabbits who succumb to the late-death syndrome and those that survive this period is presented



FIGURE 7 Thymus of rabbit ER 42 (longer-survival group) The architecture and cellularity of this thymus resemble those of a normal animal. Compare with FIGURE 3 Hematoxylin and eosin ×110

In this small series, the late-death syndrome is characterized by diffuse atrophy of lymphoid tissue and a relative plasmacytosis in an otherwise depleted spleen Grafted animals that survive this critical period have essentially normal patterns of lymphoid tissue

The possibility of employing morphological, hematological, and immunological parameters to define, as well as to analyze, the late death syndrome is implied This would have particular application to the distinction between postmature early death and premature "late" death

*Acknowledgments*

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FIGURE 8 Appendix of rabbit ER 42 (long-survival group) Lymphocytes are abundant in the lamina propria, and there are lymphoid follicles in the submucosa, which is normal Compare with FIGURE 4 Hematoxylin and eosin ×70

## *References*


1 COLE, L J, J G HABERMEYER & V P BOND 1955 Recovery from acute radiation injury in mice following administration of rat bone marrow J Natl Cancer Inst **16:** 1

2 GENGOZIAN, N & T MAKINODAN 1956 Antibody response of lethally X-irradiated mice treated with rat bone marrow J Immunol **77** 430

3 TRENTIN, J J 1957 Induced tolerance and "homologous disease" in X-irradiated mice protected with homologous bone marrow Proc Soc Exptl Biol Med **96**· 139

4 CONGDON, C C & I S URSO 1957 Homologous bone marrow in the treatment of radiation injury in mice Am J Pathol **33** 749

5 PORTER, K A 1957 Effect of homologous bone marrow injections in X-irradiated rabbits Brit J Exptl Pathol **38:** 401

6 PORTER, K A 1957 Transplantation Bull **4:** 129

7 PORTER, K A. & J E MURRAY 1958 Long term study of X-irradiated rabbits with bone marrow homotransplants J Natl Cancer Inst **20:** 189

# A STUDY OF THE GRAFT-VERSUS-HOST REACTION IN TRANSPLANTATION TO EMBRYOS, $F_1$ HYBRIDS, AND IRRADIATED ANIMALS*

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If a cellular graft contains cell members that are potentially capable of antibody formation, there are, in principle, two ways in which immunity can develop: the host can react against the graft or the latter can react against the host. The existence of this mutual reactivity was originally postulated on the basis of whole-kidney transplantation in dogs, although not formally proved at the time.[1]

An important impetus to recent studies of graft-versus-host reactions has undoubtedly been the realization of their importance for the treatment of irradiated subjects by transplantation of bone marrow. Apart from this, however, two other situations provide excellent opportunities for the study of graft-versus-host reaction: (1) transplantation to embryos or newborns, and (2) transplantation to $F_1$ hybrids from either parental strain.

With intravenous injection of adult chicken spleen cells into chicken recipients shortly before hatching, the characteristic outcome is a severe immune-hemolytic anemia and death of the host during the first or second week after hatching. These events are preceded by an intense colonization of such host organs as spleen and bone marrow with the donor's reticuloendothelial cells which, by multiplying in these sites, lead to dramatic changes such as an enormous splenomegaly. If the same kind of transplantation is performed within the first twenty-four hours after hatching of the young recipient, the consequences are mitigated insofar as mortality is lowered, but the spleen enlargement produced is still very impressive. The liver also is usually enlarged, at least partly because of multiplication of donor cells that have settled in periportal tracts of that organ.[2]

I stress these gross changes in spleen and liver because, on the basis of similar changes in mice, a new method, described below, has been developed for determining the degree of histocompatibility between different strains of mice.

## *A New Assay of Histocompatibility*

In explaining this method I shall consider the immunological situations involved in homotransplantation of spleen cells between members of $F_1$ crosses and their parental strains.

Under these conditions FIGURE 1 summarizes the theoretical possibilities for induction of immunity and tolerance to transplantation antigens that are determined by either dominant or recessive genes, provided the donor is adult

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NO ANTIGENIC STIMULUS | IMMUNITY VERSUS α ACQUIRED
ADULT DONOR
+a +b (A×B) $F_1$ −α −β
TR ANTI-α
+a AA +α
EMBRYO-NEWBORN HOST
TOLERANCE OF b INDUCED | NO ANTIGENIC STIMULUS
DOM GENES RECES. GENES

NO ANTIGENIC STIMULUS | IMMUNITY OF α ACQUIRED
ADULT DONOR
+a +b (A×B) $F_1$ −α −β
ANTI-b TR ANTI-α
+a AA +α
YOUNG-ADULT HOST
IMMUNITY OF b ACQUIRED | NO ANTIGENIC STIMULUS
DOM GENES RECES. GENES

IMMUNITY VERSUS b ACQUIRED | NO ANTIGENIC STIMULUS
ADULT DONOR
+a AA +α
ANTI-b TR
+a +b (A×B) $F_1$ −α −β
EMBRYO-NEWBORN HOST
NO ANTIGENIC STIMULUS | TOLERANCE OF α INDUCED
DOM GENES RECES GENES

IMMUNITY VERSUS b ACQUIRED | NO ANTIGENIC STIMULUS
ADULT DONOR
+a AA +α
ANTI-b TR ANTI-α
+a +b (A×B) $F_1$ −α −β
YOUNG-ADULT HOST
NO ANTIGENIC STIMULUS | IMMUNITY VERSUS α ACQUIRED
DOM GENES RECES GENES

FIGURE 1 Immunogenetic possibilities to be considered in the transplantation of antibody-forming cells between $F_1$ hybrids and their parental strains Upper left antigenic stimulation of donor cells, provided that recessively determined antigens do in fact exist, if so, splenomegaly should result Lower left stimulation of donor cells from dominantly determined antigens, the resultant splenomegaly should be a measure of the potency of these antigens Upper right immunity developed in both graft and host, host is likely to eliminate the graft, and no more than slight and transient splenomegaly should occur Lower right immunity developed in both graft and host, splenomegaly is likely to be marked if recessively determined antigens are weak in comparison with their dominant counterparts

(if donors were embryonic or newborn, four more quadrants would complete the picture)

The lower left quadrant shows the grafting of adult spleen cells of an inbred strain to embryonic or newborn recipients of an $F_1$ cross between the donor strain and another inbred strain Dominantly determined antigens corresponding to strain-specific genes A and B are tabulated as *a* and *b*, respectively Recessively determined antigens belonging to the same strains are denominated α and β It thus appears that, whereas the host can at most be stimulated by a recessively determined antigen and is, moreover, rendered tolerant of it, the donor cells meet in their new environment the dominantly determined antigen *b* and the host is thereby rendered immune against it

Since reticulo-endothelial cells that are developing immunity are at the same

TABLE 1
INDEX OF GRAFT REACTION
AKR → (AKR × DBA)$F_1$
adult 9 days (one litter)

| | Body wt (gm ) | | Spleen wt (gm /100 gm ) | | Liver wt. (gm /100 gm ) | |
|---|---|---|---|---|---|---|
| Ctr | 7 9<br>6 9 | | 0 390<br>0 378 | | 5 35<br>5 30 | |
| Mean | 7 4 | | 0 384 | | 5 33 | |
| | Gm | Index | Gm /100 gm | Index | Gm /100 gm | Index |
| Exp | 7 7<br>7 8<br>6 8 | 1 04<br>1 05<br>0 92 | 1 115<br>1 180<br>1 045 | 2 91<br>3 08<br>2 72 | 6 85<br>7 70<br>7 05 | 1 29<br>1 45<br>1 32 |

time multiplying, and since the transplanted spleen cells grow particularly well in the spleen, an enlargement of the host spleen is therefore to be expected due to the antihost attack of the donor cells that have settled there

A very significant enlargement of the spleen is actually what has been found in most of the strain combinations tested thus far TABLE 1 is a concrete example of such an experiment, conducted so that approximately one half of the 9-day-old hybrid litter is injected intraperitoneally with about 10 million nucleated cells from an adult AKR donor, after 10 days the whole litter is sacrificed and the body, spleen, and liver weights are individually recorded Spleen and liver weights are expressed as percentages of body weight and, for each experimental animal, 3 figures, so-called indices of graft reaction, are calculated These indices are determined very simply as the weight of the body or relative weight of spleen and liver over the mean of the respective weights of the littermate controls It is important that controls always be littermates because there is great variation in these values between litters and considerably less within any given litter In this particular case we can therefore conclude that the DBA strain contains at least one dominantly determined transplantation antigen that is lacking in AKR mice, and that can stimulate AKR cells to multiplication

This simple quantitative method of determining the graft-versus-host reaction has been applied in our laboratory to 5 inbred strains of mice, giving 10 possible $F_1$ crosses and, consequently, 20 different possibilities of transplantation from parental strain to $F_1$ hybrid

TABLE 2 records the results of this investigation It appears that in most combinations there is a marked increase of spleen- and liver-weight index Often this is accompanied by some reduction in body weight, but this is far from invariably the case However, there are 4 combinations involving 2 crosses in which neither parental strain produces enlargement of spleen or liver In these 2 crosses, therefore, the 2 components must be much more antigenically similar than in the remaining 8 crosses or, to put it on more exact genetic terms, AKR and C3H (of our sources at least) contain the same dominantly

TABLE 2

TRANSPLANTATION OF ADULT PARENTAL SPLEEN TO INFANT $F_1$-HYBRID

| Recipient | Donor | No. | Indices of graft reaction | | |
|---|---|---|---|---|---|
| | | | Body wt. (gm.) | Spleen wt. (gm.) | Liver wt. (gm.) |
| ST × C3H | C3H | 10 | 0.89 ± 0.10 | 2.73 ± 0.55 | 1.54 ± 0.13 |
| ST × C3H | ST | 7 | 0.88 ± 0.06 | 1.97 ± 0.34 | 1.78 ± 0.25 |
| ST × DBA | DBA | 12 | 0.98 ± 0.09 | 2.24 ± 0.35 | 1.64 ± 0.24 |
| ST × DBA | ST | 24 | 0.89 ± 0.15 | 2.40 ± 0.91 | 1.74 ± 0.32 |
| ST × DLB | DLB | 13 | 0.99 ± 0.09 | 1.91 ± 0.63 | 1.31 ± 0.30 |
| ST × DLB | ST | 14 | 0.89 ± 0.11 | 2.73 ± 0.84 | 1.58 ± 0.24 |
| ST × AKR | AKR | 10 | 0.99 ± 0.05 | 1.67 ± 0.31 | 1.38 ± 0.13 |
| ST × AKR | ST | 10 | 1.01 ± 0.04 | 3.00 ± 0.58 | 1.28 ± 0.13 |
| AKR × DBA | DBA | 13 | 1.01 ± 0.06 | 1.98 ± 0.25 | 1.34 ± 0.11 |
| AKR × DBA | AKR | 6 | 0.97 ± 0.14 | 2.68 ± 0.25 | 1.37 ± 0.06 |
| AKR × DLB | DLB | 5 | 0.85 ± 0.16 | 1.60 ± 0.44 | 2.13 ± 0.31 |
| AKR × DLB | AKR | 2 | 0.88 ± 0.17 | 1.98 ± 0.39 | 1.65 ± 0.01 |
| AKR × C3H | C3H | 12 | 1.01 ± 0.04 | 1.09 ± 0.10 | 0.96 ± 0.07 |
| AKR × C3H | AKR | 6 | 1.01 ± 0.05 | 0.98 ± 0.09 | 1.03 ± 0.06 |
| C3H × DLB | DLB | 6 | 0.95 ± 0.08 | 2.37 ± 0.55 | 1.74 ± 0.46 |
| C3H × DLB | C3H | 7 | 0.75 ± 0.15 | 2.25 ± 1.05 | 1.97 ± 0.19 |
| C3H × DBA | DBA | 3 | 1.13 ± 0.04 | 1.85 ± 0.24 | 1.16 ± 0.05 |
| C3H × DBA | C3H | 2 | 1.03 ± 0.02 | 3.81 ± 0.11 | 1.52 ± 0.04 |
| DLB × DBA | DBA | 6 | 1.07 ± 0.08 | 0.97 ± 0.09 | 0.95 ± 0.04 |
| DLB × DBA | DLB | 4 | 1.05 ± 0.05 | 1.15 ± 0.11 | 0.90 ± 0.06 |

determined transplantation antigens. The same reasoning applies to DBA and DLB.*

The recipients considered in TABLE 2 ranged from 0 to 14 days of age at the time of transplantation. Within this age range a more detailed analysis (which is to be made when more facts are available) will probably show that enlargement of the liver and spleen in a given donor-host combination is, to some extent, dependent on the age of the host. Most noticeably, there is a marked tendency for enlargement of the liver to be the more pronounced the younger the host.

### *Application of the Graft-Versus-Host Assay of Histocompatibility*

If the above assay is valid, it should follow that, after lethal irradiation, any one of these 4 strains could be treated successfully with spleen homotransplantation from the other strain with which it proved compatible, but not with any of the other strains available.

The testing of this prediction is in its inception, and TABLE 3 gives preliminary results. The table gives the aggregated data from 2 identical experiments in which 2- to 3-month-old C3H mice were X-rayed by continuous exposure for 24 hours with a total dose of 1100 r. As seen from the table this dose gave a 30-day survival of 15 per cent in noninjected controls, the peak of mortality appearing about 2 weeks after irradiation. However, the 2 groups that had received some 50 million nucleated adult spleen cells intravenously in the first

* Actually, DLB and DBA, as employed here, represent 2 different substrains of the DBA strain imported from the United States in 1938 and 1952 respectively.

TABLE 3
TRANSPLANTATION OF ADULT SPLEEN CELLS TO C3H MICE
IRRADIATED WITH 1100 R IN 24 HOURS

| Donor | Number of deaths Days after transplantation | | | | | | | | | | | | Alive at 30 days |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | |
| Nil | | | | 1 | | 1 | 2 | 2 | 6 | 1 | 3 | 1 | 3/20 |
| AKR | | 1 | 1 | 1 | | | | 1 | | 1 | 2 | | 13/20 |
| ST | 1 | 6 | 12 | 2 | | 1 | | | | 1 | 1 | | 0/24 |

few hours after irradiation behaved quite differently Those that received cells of the AKR strain had a 65 per cent survival, whereas all 24 mice injected with ST cells died, most of them as early as one week after irradiation Thus there is no doubt that, in perfect agreement with the prediction, AKR cells are helpful to irradiated C3H mice, while ST cells make matters worse It still remains to be seen if the prediction holds equally true for transplantation of cells from DLB and DBA

I believe it is fair to say that this method for determining histocompatibility has three merits in comparison with previous ones First, it is much the simplest to perform when inbred strains are already available Second, it gives a direct answer to investigators who are especially concerned about transplantation of reticuloendothelial cells, whereas transplantation of skin or tumors, like a detailed blood group analysis, might for this purpose very well reveal either too much or too little incompatibility Third, the method is likely to be a handy tool for testing the efficiency of any possible procedure that aims at preventing or diminishing the immune response of reticuloendothelial cells

Under the latter heading, of course, would come the induction of tolerance TABLE 4 shows a preliminary attempt to learn how long after birth reticuloendothelial cells of C3H origin will still be able to become tolerant instead of immune from the contact with dominantly determined transplantation antigens of ST origin

This table consists of two parts In its lower section spleen and liver indices show the strong immune reaction of adult spleen cells transplanted to newborn or infant $F_1$ hybrids Even in fully mature hybrid recipients there is a clear-cut increase of the spleen index

In contrast to this, the upper part of the table records the results from transplantation of infant C3H spleen of 6 to 13 days of age In this case the indices point at tolerance because they deviate quite insignificantly from 1 00 in spite of the fact that the litters were not sacrificed until after 14 or 28 days in order not to miss an immunity that might well be delayed until the young donor cells had matured sufficiently to become immune in any degree

It remains to be seen if infant C3H cells that prove tolerant of ST antigens as measured in this way will also be able to protect irradiated ST mice

Not least in importance for the treatment of leukemia by heavy irradiation,

TABLE 4
ACQUIRED TOLERANCE IN INFANT SPLEEN GRAFTS

| Donor C3H | Host (C3H × ST)$F_1$ | Indices of graft reaction | | | | | |
|---|---|---|---|---|---|---|---|
| | | Body wt. | | Spleen wt | | Liver wt | |
| | | Sacrificed 14 or 28 days after transplantation | | | | | |
| Age | Age | 14 days | 28 days | 14 days | 28 days | 14 days | 28 days |
| 6 or 7 days | 2 or 3 days | 1 00<br>0 96 | 1 02<br>0 87<br>0 98<br>0 89<br>0 89<br>1 01 | 0 75<br>0 97 | 0 98<br>0 86<br>0 89<br>0 77<br>1 17<br>1 12 | 1 00<br>1 03 | 1 00<br>1 01<br>0 99<br>1 08<br>0 96<br>0 98 |
| 13 days | 8 or 9 days | 0 97<br>1 18 | 0 97 | 0 85<br>1 15 | 1 03 | 1 07<br>1 17 | 0 99 |
| | | Sacrificed 10 days after transplantation | | | | | |
| Adult | 0–5 days | 0 89 ± 0 10 | | 2 73 ± 0 55 | | 1 54 ± 0 13 | |
| Adult | 18–23 days | 0 92 ± 0 08 | | 2 58 ± 0 48 | | 1 13 ± 0 09 | |
| Adult | 63–83 days | 1 00 ± 0 06 | | 1 48 ± 0 50 | | 1 06 ± 0 11 | |

it is essential to find ways of either selecting donors that are naturally compatible with the host or inducing tolerance in the cells of those that are not naturally so At present I can think of no project likely to be more promising than transplantation from fetal, newborn or, possibly, even infant donors

Later experiments have provided the following data (1) Adult spleen cells from DLB and DBA mice are useless as adult ST cells for the treatment of lethally irradiated C3H mice (2) Most of the 30-day survivors shown in TABLE 3 that were treated with adult AKR spleen have later developed secondary (homologous) disease Evidence is being accumulated that this phenomenon is caused by recessively determined antigens that are not detectable by the method of assay used in TABLE 2 (3) C3H mice that received an $LD_{100}$ dosage of X rays, followed by spleen cells from infant AKR mice from 6 to 13 days of age have all survived for 30 days and, with few exceptions, are still alive after 2 months without exhibiting any signs of secondary disease (4) Similar attempts to rescue C3H mice by the use of spleens from infant ST, DBA, and DLB mice have resulted in the massive development of fatal secondary disease

It thus appears that tolerance is more solidly acquired to transplantation antigens that are determined by recessive genes than to those determined by dominant genes

*References*


1 SIMONSEN, M 1953 Biological incompatibility in kidney transplantation in dogs Acta Pathol Scand **32:** 36–86

2 SIMONSEN, M 1957 The impact on the developing embryo and newborn animal of adult homologous cells. Acta Pathol Scand **40**· 480–500

*Discussion of the Paper*

A A LAZZARINI (*New York University Post Graduate Medical School, New York, N Y*) With reference to Simonsen's interesting findings of splenomegaly in chickens in our studies of embryonal parabiosis in birds we found that if the procedure was done rather late, that is, after the sixteenth day of incubation, some of the parabiotic mates became weak after hatching, were underdeveloped, and died in the following four weeks It was common to find splenomegaly and multiple infarctions in the weak member of the team just before its death

Perhaps some cases of so-called parabiotic intoxication that we have observed were due to a graft-to-host reaction similar to that demonstrated by Simonsen and Billingham

W H HILDEMANN (*University of California Medical Center, Los Angeles, Calif*) One of the proofs of the immunological basis of graft-versus-host reactions, as given in this monograph by Billingham, Simonsen, and Trentin, has come from the inoculation of newborn or lethally irradiated adult animals from either of two inbred lines with adult spleen cells or bone-marrow cells derived from their $F_1$ hybrid In this situation, under appropriate conditions no graft against host incompatibility is expected, since all histocompatibility antigens of both parent lines are present in the $F_1$ hybrid cells In the reciprocal situation, of course, incompatibility would be expected, depending on the antigenic constitutions of the strains tested The evidence presented today generally fulfills these expectations and indirectly supports the "one-autosomal gene, one-antigen" theory

However, if members of the $F_1$ hybrid between any two pure lines of animals possessed more or less than the total antigens of the parent lines, the adult graft-versus-tolerant-host system could reveal histocompatibility-gene interaction and would be affected by it as follows

(1) If a hybrid antigen(s) were produced as a consequence of gene interaction in the $F_1$ hybrid, and the interacting genes thereby failed to produce a histocompatibility antigen(s) found in the homozygous parent line(s), a reaction of hybrid lymphoid cells against the cells of a tolerant, parental strain host should occur One would also expect a parent line graft to be unacceptable to an $F_1$ hybrid normal adult host under these circumstances However, the presence of hybrid antigens in addition to all parent-line antigens in the $F_1$ hybrid would elicit no graft incompatibilities in these test systems

(2) If genes in the $F_1$ hybrid between two highly inbred lines interacted so as to suppress the production of a histocompatibility antigen found in one or both of the parent lines, hybrid graft against tolerant, parent-line host as well as hybrid normal host against parent-line graft reactions should be detectable as above

(3) If a histocompatibility antigen in one parent-line were determined by a recessive pair of genes, as suggested by Simonsen, one might also expect $F_1$ hybrid cells to react against a tolerant parent-line host possessing the antigen This possibility could be tested readily by placing skin grafts from the parent lines on $F_1$ hybrid adult recipients The $F_1$ hybrid should reject skin grafts

from one of the two parent lines if a recessively determined antigen is present in a graft

Although none of these possibilities is apparently supported by homograft studies performed so far, further investigation is desirable Haldane[1] has called attention to instances in which hybrid antigens have been demonstrated and to several methods for the detection of antigens with an abnormal genetic determination Several recent studies, notably those of Ceppellini,[2] Rendel *et al*,[3] Levine *et al*,[4] Cohen,[5] and others, have demonstrated gene interaction in the production of hybrid antigens or the suppression of parental antigens within various mammalian species Since it is now obvious that the one gene-one antigen relationship does not hold true for all situations, a closer study of histocompatibility gene-antigen relationships is indicated

*References*


1 HALDANE, J B S 1956 The detection of antigens with an abnormal genetic determination J Genet **54:** 54–55
2 CEPPELLINI, R 1954 On the genetics of secretor and Lewis characters a family study 5th Intern Congr Blood Transfusion Paris, France
3 RENDEL, J, A N SORENSEN & M R IRWIN 1954 Evidence for epistatic action of genes for antigenic substances in sheep Genetics **39:** 396–408
4 LEVINE, P, E ROBINSON, M CELANO, O BRIGGS & L FALKINBURG 1955 Gene interaction resulting in suppression of blood group substance B Blood **10** 1100–1108
5 COHEN, C 1956 Occurrence of three red blood cell antigens in rabbits as the result of interaction of two genes Science **123.** 935–936


JOHN J TRENTIN (*Baylor University College of Medicine, Houston, Texas*) Simonsen has mentioned the interesting possibility that grafted reticuloendothelial cells may be rendered tolerant of their homologous host provided they are derived from sufficiently young donors Theoretically, it should be possible to avoid homologous disease by doing such a "fetal tolerance in reverse" With this in mind I have compared fetal liver with adult bone marrow for protection of lethally irradiated mice in six combinations of homologous donor and recipient strains Fetal liver is hematopoietically active and does protect after lethal irradiation but, to my disappointment, it offered no significant advantage over adult marrow with respect to the occurrence of homologous disease

# ACQUIRED TOLERANCE TO HOMOGRAFTS AND HETEROGRAFTS IN THE RAT*

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## *Introduction*

In a preliminary report[1] we described our initial experiences in attempting to produce heterologous acquired tolerance in mammals The purpose of this paper is to present a more detailed description of heterologous tolerance experiments, as well as some recent work on homologous acquired tolerance Confirmation of Woodruff's[2] production of homologous acquired tolerance in rats has not been published to date, although the principle of acquired homologous tolerance in mice described initially by Billingham *et al* [3] has now been established and confirmed

It is now apparent that the species used is of great importance Marked differences in the ability to achieve the status of acquired tolerance exist in mammals Furthermore, although acquired tolerance to several types of antigens such as homografts, bacterial products, and tumors may be produced, it appears that different injection times are necessary in different animal species It will no doubt take several years to clarify the whole antigen exposure-response relationship in producing acquired tolerance in the various laboratory animals and man The rat was chosen for the present study because of the immaturity of the newborn, hardiness of the mother when operated on, and lack of a close genetic similarity between rats of different litters

## *Methods*

White rats† were used in these experiments New Zealand white rabbits approximately 4 pounds in weight donated the heterologous spleen and skin We also used C3H mice ‡ Splenectomy was performed, using sterile technique and a midline abdominal incision in the rabbit and mouse The short- and long-term survivals in these splenectomized animals were excellent, and we observed an interesting phenomenon in the rat spleen donors used in the homologous experiments Removal of the entire spleen led to a mortality of 80 to 95 per cent within 1 week to 2 months This death rate was cut down to 10 to 20 per cent when 2 end fragments of the rat spleen were retained and the central portion only was excised We have no explanation of the basis for this increased mortality in the totally splenectomized animals as compared to those with small fragments of the spleen remaining

Splenic cell preparations were made in a manner designed to yield the highest possible number of viable and intact cells The cells were gently teased out

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† Obtained from Holtzman Co , Madison, Wis

‡ Provided by Carlos Martinez, Department of Physiology, University of Minnesota

of the spleen into a saline medium to which a drop of heparin had been added Counts were made of this suspension Also they were stained with trypan blue to identify the percentage of viability In the homologous experiments, such cell suspensions were injected either intraperitoneally or intravenously in the median facial vein of the newborn For the heterologous experiments, pregnant rats were operated on through a midline lower-abdominal incision, and the uterine horn bearing the fetus was gently lifted from the abdominal cavity By means of a No 30 needle, all fetuses were injected with 0 01 cc cell suspension, the uterine horns were replaced, and the abdominal wall was closed As an alternative to the cell suspension, a cell brei of tissue was prepared by gently mashing the spleen after removal of the capsule This brei was injected intraperitoneally through a small trocar into newborns When these animals had attained an age of at least 6 weeks, skin grafts were applied according to a technique described elsewhere [4] The status of the grafts was determined by sequential observation of intradermal blood flow,[5] removal rate of intradermally injected fluorescein,[4] and occasional biopsy In a few instances, regrafting of a tolerant homograft back onto the original cell donor was performed according to the usual method

## *Results*

*Acquired tolerance to homografts in rats* Reference to TABLE 1 demonstrates that homologous tolerance to skin in rats is achieved more frequently by the injection of a cell brei than by the use of a cell suspension All except a few litters of animals so prepared were injected within a few hours of birth However, 5 2-day old rats were injected with homologous cells, 4 were found to be tolerant when grafted at 2 months of age There is one very important difference between the cell suspension and brei groups only about one third of the newborn rats injected with cell brei survived for the 2 months necessary for grafting, in contrast to more than two thirds of the number of newborns injected with cell suspension At necropsy a relative lack of lymphoid tissue was noted in many of the brei-treated animals that were not normally active, had shaggy coats, and did not gain weight normally

Three regrafting experiments in which grafts were replaced in the original cell donor after they had been in place in the tolerant host for 3 months were performed Such regrafts were not rejected by the cell donor, but persisted as autografts

*Heterologous acquired tolerance* Most of these experiments involved the rabbit-to-rat heterograft system in which it had previously been determined that these transplants survived for an average time of 5 9 days In a control series of more than 70 rabbit-to-rat grafts, none survived for longer than 8 days

TABLE 1

| No of rats | Type of preparation injected | No of viable cells injected | Per cent tolerance |
|---|---|---|---|
| 37 | Cell suspension | 99 to 350 Million | 63% |
| 33 | Cell brei | Not counted | 86% |

TABLE 2

| No of rats | Heterologous donor | Injection time, days before birth | "Reinforcement" cell injection after birth | No of cells (millions) given I P | Survival times |
|---|---|---|---|---|---|
| 2 | Rabbit | 4 | No | 9 5 | 17 and 13 |
| 2 | Rabbit | 2 | No | 20 0 | 11 and 14 |
| 2 | Mouse | 1 | No | 7 0 | 16 and 19 |
| 1 | Rabbit | 3 | No | 8 0 | 23 |
| 2 | Rabbit | 3 | No | 6 0 | 13 and 16 |
| 2 | Rabbit | 2 | First and fourth days | 22 0 | 11 and 13 |
| 3 | Rabbit | 2 | First and third days | 37 0 | 11 and 14 |

TABLE 2 shows the results of experiments directed toward the production of heterologous acquired tolerance in rats It can be seen that a significant prolongation of survival was obtained in several rabbit- and mouse-to-rat heterografts by injecting the fetuses *in utero* with cells from the future heterologous host In control studies animals were grafted from a heterologous source other than the original donor, and in these cases no prolongation beyond normal survival time for standard type heterografts was observed

"Reinforcement" of rats which had been previously injected *in utero* with rabbit cells by a second injection of cells from lymph nodes of the same donor rabbit in the neonatal period resulted in a few prolongations of graft survival beyond the expected period—a greater percentage than was encountered with the *in utero* injections alone In all these heterologous experiments it is to be emphasized that the fetal mortality was quite high, ranging from 60 to 80 per cent Moreover, of those rats injected *in utero* that reached term, only about 10 to 15 per cent were partially tolerant of skin from the original heterologous donor Despite improvements in surgical techniques and careful postoperative management at all stages, this high fetal and neonatal mortality has not been reduced A higher percentage of partially tolerant heterografts was not found in those animals injected 4 days before delivery, as contrasted with those injected 1 day before birth No prolongation of heterograft survival was obtained by injecting heterologous cells into the neonates in the absence of prior *in utero* injection

However, it appeared quite clear that the animals that received "reinforcement" doses of cells in the neonatal period in addition to *in utero* injection had a larger percentage of moderate prolongations of survival when compared to the control series Only an occasional such "booster" animal rejected the heterograft before 8 days, and most were in the 9- to 11-day range So, although repeated cell injection in the neonatal period did not result in indefinite heterograft survival, it did slightly increase the survival period of the majority of them

## *Discussion*

The present studies fully confirm Woodruff's findings of acquired tolerance to skin homografts in rats[2,6] Recent work by Billingham *et al*[7] indicates

that a higher degree of acquired tolerance is produced in newborn mice by the intravenous injection of cells and that the intraperitoneal and subcutaneous routes are less effective in this regard It appears that the rat is an easy experimental animal in which to produce acquired tolerance to homografts, but we do not know whether tolerance to other antigens is produced as readily Our data demonstrating partial heterologous tolerance in the rat to rabbit and mouse skin would reinforce this concept Although we were unable to achieve long-term survival of skin heterografts, several grafts exhibited intradermal blood flow for several days after the usual rejection time

The "runt" syndrome has been described by several groups of workers,[8-10] both in connection with the attempts at the production of tolerance and associated with whole-body radiation and with intravenous homologous or heterologous bone marrow Such stunted animals have a deficiency of lymphoid tissue at death, and it has been theorized that such a state results from a "graft-versus-host" reaction Although this study sheds no light on the mechanism, we believe that a high percentage of newborn rats injected with homologous cell brei were victims of the runt syndrome The work of Billingham and Brent[8] suggests that this defect can be prevented by the use of cells that are not of the reticuloendothelial system Confirmation of this concept would have great practical significance in the development of acquired tolerance in clinical work [11, 12]

That a brei of the spleen was more effective in our studies in producing tolerance than cell suspensions was most likely due to the injection of greater numbers of viable cells in the former experiments It also became clear early in our experience that even large numbers of nonviable cells were ineffectual in producing tolerance This observation is supported by the finding of Billingham *et al* that the injection of subcellular particles[13] was not effective in this regard This would appear to strengthen the arguments that such cells survive in the host as long as acquired tolerance itself persists Billingham and Brent[7, 8] found that an injection of spleen from a nontolerant mouse would so alter the response of tolerant mice that subsequent skin grafts derived from the original cell donors would be rejected after the manner of a second-set graft

That a skin graft in place in a homologous host for more than three months can be regrafted to the original donor has important implications Seemingly this transplanted skin has maintained its original identity despite prolonged residence in a foreign environment Good *et al* found a female chromatin pattern in a skin homograft that had been in place on an agammaglobulinemic boy for over two and one half years [14] These experiments would not strengthen Woodruff's concept of the "critical period" According to experiments on guinea pigs published by the Woodruffs in 1950,[15] homologous thyroid that survived for over three months in the anterior chamber of the eye could then be successfully transplanted to other sites in which rejection had formerly occurred Presumably, alterations of the homograft tissue occurred with time, and the new tissue was no longer foreign to the host This concept of the critical period has not been confirmed to date, unless one is willing to accept Woodruff's[6] rejection of new homografts in partially tolerant rats with surviving grafts from an earlier transplantation as such We conclude from our

studies that the individuality of cells persists in a foreign host even after long periods of time This hypothesis is strengthened by the ingenious experiments of Billingham *et al*[16] in which normal lymph nodes were transplanted from a nontolerant mouse to a tolerant mouse of the same strain bearing a homograft, the tolerant homograft was subsequently rejected in these cases A logical reason for this rejection would depend on reactive specific antigenic material from the homograft reaching those reactive transplanted lymph nodes from the nontolerant source

With the methods of this study we were unable to produce the long-term heterologous tolerance obtained by Zaalberg *et al* using total-body radiation and heterologous bone marrow,[17] in which rat skin heterografts survived for at least 128 days, and mice and rat serum proteins[18] were shown to be continually produced by the mouse These findings confirm the fact that heterograft survival is a possibility It is likely, however, that this type of success will be secured more easily with rat-mouse combinations than with rabbit-rat pairings Technical reasons preclude the injection of cells *in utero* at a much earlier date than was performed in these experiments, and it is by no means established that the "earlier the better" principle pertains in tolerance production Our studies are continuing with various antigenic preparations in an attempt to produce a longer lasting heterologous tolerance in a greater percentage of animals

## *Conclusions*

(1) Acquired tolerance to homologous skin is easily produced in more than 80 per cent of rats by the neonatal injection of cells from the prospective donor

(2) Use of large numbers of homologous cells in the newborn inoculum results in a high incidence of the "runt syndrome"

(3) Homografts resident for long periods in tolerant rats can be successfully regrafted onto the original donor animal

(4) Partial heterologous tolerance to rabbit skin was produced in rats by injection of rabbit cells *in utero*

## *References*


1 EGDAHL, R. H & R L VARCO 1957 Heterologous tolerance in mammals Transplantation Bull **4** 72

2 WOODRUFF, M F A & L O SIMPSON 1955 Induction of tolerance to skin homografts in rats by injection of cells from the prospective donor soon after birth Brit J Exptl Pathol **36.** 494–499

3 BILLINGHAM, R E, L BRENT & P B MEDAWAR 1953 Actively acquired tolerance of foreign cells Nature **172.** 603–605

4 EGDAHL, R H, R A GOOD & R L VARCO 1957 Studies in homograft and heterograft survival Surgery **421** 228–239

5 TAYLOR, A C & J W LEHRFELD 1953 Determination of survival time of skin homografts in the rat by observation of vascular changes in the graft Plastic Reconstr Surg **12:** 423–431

6 WOODRUFF, M F A 1957 Postpartum induction of tolerance to homologous skin in rats Ann N Y Acad Sci **64**(5)· 792–799

7 BILLIGHAM, R E & L BRENT 1956 Acquired tolerance of foreign cells in newborn animals Proc Roy Soc London **B145** 78–90

8 BILLINGHAM, R E & L BRENT 1957 A simple method for inducing tolerance of skin homografts in mice Transplantation Bull **4** 67–71

9 SCHWARTZ, E E, A C UPTON & C C CONGDON 1957 A fatal reaction caused by implantation of adult parenteral spleen tissue in irradiated $F_1$ mice Proc Soc Exptl Biol Med **96:** 797–800

10 WOODRUFF, M F A & M SPARROW 1957 Further observations on the induction of tolerance of skin homografts in rats Transplantation Bull 4 157–159

11 WOODRUFF, M F A 1957 Can tolerance to homologous skin be induced in the human infant at birth? Transplantation Bull **4:** 26–28

12 PEER, L 1956 Long survival time of skin graft from mother to male child (with biopsy section of skin graft taken 20 days after transplantation) Plastic Reconstr Surg **18:** 169–176

13 BILLINGHAM, R E, L BRENT & P B MEDAWAR 1956 The antigenic stimulus in transplantation immunity Nature **178.** 514–519

14 GOOD, R A, R L VARCO, J B AUST & S J ZAK. 1957 Transplantation studies in patients with agammaglobulinemia Ann N Y Acad Sci **64**(5). 882–928

15 WOODRUFF, M F A & H G WOODRUFF 1950 The transplantation of normal tissues with special reference to auto- and homotransplantation of the thyroid and spleen in the anterior chamber of the eye, and subcutaneously, in guinea pigs Phil Trans Roy Soc London **B234.** 559–582

16 BILLINGHAM, R E, L BRENT & P B MEDAWAR 1956 Quantitative studies on tissue transplantation immunity III Actively acquired tolerance Phil Trans Roy Soc mice London **B239** 357–414

17 ZAALBERG, O B, O VOS & D W VAN BEKKUM 1957 Surviving rat skin grafts in Nature **180** 238–239

18 WEYZEN, W W H & O VOS 1957 Production of rat serum proteins in irradiated mice Nature **180** 288–289

# THE USE OF GLOBULINS AS A MEANS OF INDUCING ACQUIRED TOLERANCE TO PARABIOTIC UNION*

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## *Introduction*

This preliminary report deals with the parabiotic method of analyzing tissue homotransplantation compatibility This method of transplantation was first conceived by Paul Bert[1] and actually developed as a technique by Sauerbruch and Heyde [2] Although animals surgically united in this way do at times go into apparent successful union, each partner maintains its own organic functions and, to some extent, its own individual metabolism It has been shown by Huff *et al* ,[3] Kamrin,[4] and other workers that the blood and tissue fluids pass continuously from one partner to the other In successful parabionts there is gross evidence of the presence of large anastomotic connections in the connective tissues joining the two animals Histological examination of the joined skin discloses continuity of the epithelium and a narrow connective tissue zone of condensed fibers perforated by numerous capillaries

The extensive literature[5, 6] on parabiotic studies does not indicate the mechanism by which successful parabiosis is attained Loeb[7] implies that the great dilution of each animal's fluids by its copartner will result in a tolerant gradient of each animal's "individuality differentials " Genetically, the work of Snell[8] and his co-workers suggests that histocompatibility genes are present in both successful parabionts In addition, the work of Eichwald *et al* [9] would indicate that the male skin rejection phenomena by female recipients of the same strain is determined by a Y-linked gene It is further suggested by the authors "that hormonal mechanisms may modify a course of events largely determined by the distribution and strength of histocompatibility genes " The numerous investigations of Billingham *et al* [10] open the possibility that "actively acquired tolerance" as a specific inhibition of response is the immunological mechanism that may favor successful parabiotic union

Unsuccessful parabiotic union is clearly manifested by (1) anemia and/or stunting of one of the parabionts, (2) death of one or both parabionts, (3) localized alopecia, (4) unhealed scar tissue in line of union, (5) formation of raw and oozing areas at or near the line of union, and (6) slow or rapid separation of the animals at the original site of surgical union When successful parabiotic union is obtained with pen-bred littermate albino rats, it is assumed that complete interanimal tolerance has been achieved Since the blood cells, plasma, and tissue fluids are the major constituents exchanged and shared between the copartners, it is believed that any changes resulting from such mutual acceptance will be reflected in the distribution of the serum proteins The present study deals with (1) the quantitative measurements of the blood serum components in single and sham-operated animals and in successfully

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parabiosed animals, and (2) the effects induced by the injection of the various globulins on the survival rate of parabiosed animals and on their electrophoretic patterns

### *Materials and Methods*

*Animals* The several hundred animals used in these experiments were pen-bred albino rats * Where possible, littermates were divided among the various groups so as to randomize the data obtained The various groups of animals were treated as follows (1) single animals, (2) sham-operated animals, (3) littermates of same sex and approximate size placed into parabiotic union, (4) littermates divided into groups that were pretreated with various globulins and others untreated, all subsequently surgically united in parabiosis, (5) littermates united into parabiosis and injected with various globulins immediately following parabiosis and at subsequent times, and (6) nonlittermates subjected to some of the injection procedures

*Sham operation* Animals aged 25 to 50 days were subjected to the incisions performed in preparing the animals for parabiotic union The abdominal and skin incisions were closed with sutures and two animals were permitted to recuperate in each cage Some of these animals were subjected to weekly and monthly bleedings to determine the electrophoretic changes that occurred during the healing process

*Parabiotic union* Usually littermate pairs of the same sex and size, aged 21 to 26 days, were surgically united in parabiosis When nonlittermate pairs were united we attempted to pair the animals by age and weight The procedure used was a modified version of the Bunster and Meyer[11] technique

*Serum analysis* Blood was obtained under direct observation from the external jugular vein of anesthetized animals Approximately 0 5 ml of blood was drawn from 21- to 25-day-old rats and 1 0 to 2 0 ml of blood from older rats After clotting, the blood was centrifuged at 0° to 4° C for 30 min at 2500 rpm

The specific gravity of the separated serum was determined by the copper sulfate falling-drop method[12] and confirmed by a micro-Kjeldahl method [13] The latter procedure was modified by prolonging the digestion with concentrated sulfuric acid to 2½ hours Both methods showed a good degree of correlation relative to the total protein content of the serum

Approximately 0 2 ml of the remaining serum was dialyzed against Veronal buffer, *p*H 8 6, ionic strength 0 10, for 90 to 150 min Before it was placed in the cell for electrophoresis the dialyzed serum was diluted to approximately 2 0 gm per 100 ml with fresh Veronal buffer Serum protein analysis was carried out by the Antweiler[14] microelectrophoresis apparatus at a constant current of 1 5 mAmp

The interference readings of the various protein concentrations were recorded on a tape that was converted to the typical schlieren pattern, and the various fractions indicated By marking the extent of each peak on the tape, direct measurements permitted calculation of the percentage of each component in

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the pattern There was infrequent separation of the alpha$_1$-globulin component, so that it was estimated as part of the albumin fraction

The absolute amounts of each component were found by multiplying the percentage by the grams/100 ml (from conversion of specific gravity readings) The albumin/globulin (A/G) ratio was obtained by dividing the percentage of albumin (plus alpha$_1$-globulin) by the total globulin percentages During the infrequent intervals when a separate peak for alpha 1-globulin was obtained it was found to be approximately 4 0 per cent of the total proteins

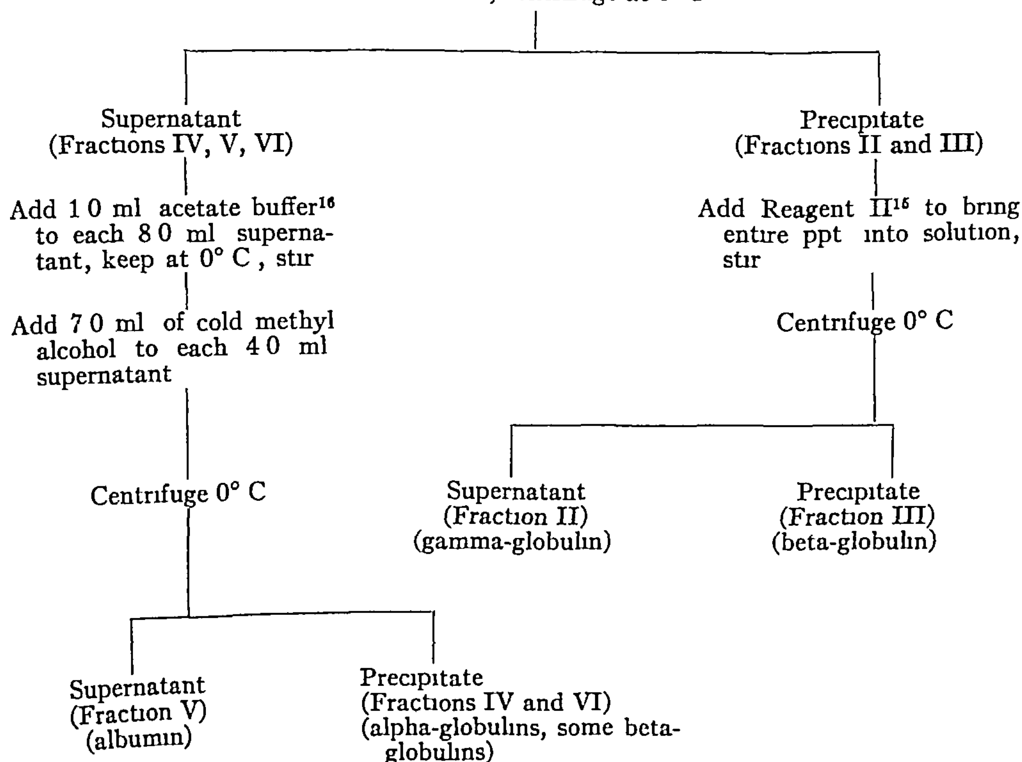
*Preparation of rat globulins* During the bleeding of the various parabiotic pairs the serum was saved and pooled according to the original treatment of the donor animals It was stored in the freezer compartment until fractionated The fractionation procedure was a combination of several methods[15, 16] and resulted in the separation of relatively homogeneous electrophoretic fractions (TABLE 1)

The procedure followed in fractionation of rat's blood serum is a combination of the Goldstein and Anderson[15] technique and that described by Pillemer and Hutchinson [16] Reagent I, referred to in TABLE 1, consists of 0 25 ml sodium acetate–acetic acid buffer (*p*H 4 0), 25 0 ml of 95 per cent ethanol, and distilled water to make 100 ml Reagent II consists of 0 1 ml of 1 0 M sodium acetate, 0 1 ml of 0 7 M acetic acid, 2 25 gm of glycine, 7 5 ml of 95 per cent ethanol,

TABLE 1

FLOW CHART OF FRACTIONATION

To each millimeter of serum add 4 0 ml of Reagent I,[15] keep at 0° C, stir 30 min, centrifuge at 0° C



and distilled water to make 50 0 ml The procedure used in separating Fraction V from the other fractions requires the use of acetate buffer The latter is made by diluting 72 ml of 1 M acetic acid and 12 ml. of sodium hydroxide to 1 liter with distilled water The methyl alcohol reagent is made by adding 607 ml methyl alcohol to 393 ml distilled water Mix, cool to 0° C., and make to 1 liter with cold methyl alcohol

The various fractions were lyophilized and stored at room temperature When needed they were dissolved in 0 15 M NaCl to make a 10 per cent solution, and 0 1 ml. (10 mg) was injected intraperitoneally After being placed into solution the globulins were maintained at a constant temperature in a refrigerator

## *Results*

*Control animals* Single and repeated serial electrophoretic studies of the blood serum of normal animals (TABLE 2) show that the specific gravity of the serum increases with age to a maximum at about 100 days The A/G ratio remains at approximately the same level throughout the period of analyses The globulin components show minor fluctuations, none of which are significant When littermates of these animals are subjected to a sham operation, significant changes are found These changes are reflected in the initial fall of the A/G ratio and the early increase in the beta-globulin component Approximately 60 days after the infliction of the wound (and about 30 days after gross healing has occurred) there is a return of the blood serum to control levels

*Successful parabiotic union* Although more than 100 pairs of littermates have been placed into surgical union, the data will deal with 43 pairs that have been carefully followed As shown in TABLE 3, only 4 pairs were found to be in successful parabiotic union The majority of animals (33 pairs) died between the second and thirty-second day of union Only 6 pairs survived sufficiently long to separate completely It is noteworthy that of the total pairs placed in union, 16 pairs were female littermates and 27 pairs were male littermates, in the successful pairs, 3 pairs were female and only 1 pair was male

Serial electrophoretic study of the blood serum of these successful pairs produced the data given in TABLE 3 In contrast with the data obtained in the sham-operated animals, the operative procedure and continuing parabiotic union result in a prolonged fall in the A/G ratio that persists for about 150 postoperative days The lower albumin values were found concomitant with increased beta-globulin levels There were no significant changes in the other globulin components

*Survival effects of protein fraction injections* The various components obtained by the fractionation procedures were utilized by (1) pretreatment of the prospective parabionts and (2) giving injections of the material immediately following parabiosis and subsequently for the same dosage in both groups TABLE 4 tabulates the results of these procedures, from this one may note that, although there were slightly more male parabiotic pairs (63) than female pairs (60), the females were more frequently found to go into successful parabiosis

TABLE 2

CONTROL DATA MEANS OBTAINED FROM 25 NORMAL ALBINO RATS

| Age range, days | No of animals | Specific gravity | A/G ratio | Albumin-$\alpha_1$ | | Alpha$_2$ | | Beta | | Gamma | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Per cent | Gm per cent | Per cent | Gm per cent | Per cent | Gm per cent | Per cent | Gm per cent |
| **Normal** | | | | | | | | | | | |
| 21 to 30 | 4 | 1 021 to 2 | 1 80 | 64 4 | 3 49 | 5 3 | 0 30 | 21 4 | 1 32 | 8 8 | 0 57 |
| 31 to 40 | 10 | 1 022 to 3 | 1 85 | 65 0 | 3 81 | 6 2 | 0 36 | 21 5 | 1 36 | 7 3 | 0 43 |
| 41 to 50 | 4 | 1 023 to 4 | 1 90 | 65 8 | 4 20 | 3 4 | 0 23 | 23 6 | 1 52 | 7 2 | 0 47 |
| 51 to 60 | 3 | 1 024 | 2 00 | 67 1 | 4 21 | 5 4 | 0 35 | 18 7 | 1 17 | 8 7 | 0 55 |
| 61 to 80 | 4 | 1 024 to 5 | 1 95 | 66 3 | 4 53 | 4 0 | 0 27 | 19 4 | 1 32 | 10 3 | 0 70 |
| 81 to 100 | 2 | 1 025 to 6 | 1 70 | 63 0 | 4 27 | 3 9 | 0 27 | 22 4 | 1 52 | 10 7 | 0 72 |
| 101 to 120 | 2 | 1 025 to 6 | 1 90 | 65 8 | 4 49 | 4 2 | 0 28 | 20 3 | 1 39 | 9 7 | 0 67 |
| **Sham-operated (at age 30 to 35 days)** | | | | | | | | | | | |
| 41 to 50 | 4 | 1 023 to 4 | 1 35 | 57 4 | 3 61 | 4 8 | 0 30 | 27 8 | 1 76 | 10 0 | 0 62 |
| 51 to 60 | 2 | 1 024 | 1 24 | 53 1 | 3 46 | 4 8 | 0 31 | 32 3 | 2 05 | 9 8 | 0 63 |
| 61 to 80 | 3 | 1 024 to 5 | 1 47 | 59 6 | 3 91 | 5 0 | 0 36 | 26 4 | 1 90 | 9 0 | 0 63 |
| 81 to 100 | 2 | 1 025 to 6 | 1 91 | 65 7 | 4 49 | 6 3 | 0 43 | 18 3 | 1 25 | 9 7 | 0 66 |
| 101 to 120 | 2 | 1 026 | 1 84 | 64 8 | 4 37 | 5 3 | 0 37 | 23 7 | 1 77 | 6 2 | 0 50 |

TABLE 3

SUCCESSFUL UNTREATED PARABIOTIC PAIRS EFFECTS OF PARABIOTIC UNION ON SURVIVAL RATE

| Total No of pairs united | | No of successful pairs | | Died during union | | Spontaneous separation | |
|---|---|---|---|---|---|---|---|
| Male | Female | Male | Female | Male | Female | Male | Female |
| 27 (62 8%) | 16 (37 2%) | 1/27 (3 7%) | 3/16 (18 7%) | 23/27 (85 2%) | 10/16 (62 5%) | 3/27 (11 1%) | 3/16 (18 8%) |

ELECTROPHORETIC ANALYSES SHOWING MEAN ABSOLUTE VALUES OF THE VARIOUS COMPONENTS

| Age range, days | No of pairs | Specific gravity | A/G ratio | Albumin a₁ | | Alpha₂ | | Beta | | Gamma | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Per cent | Gm per cent | Per cent | Gm per cent | Per cent | Gm per cent | Per cent | Gm per cent |
| 41 to 50 | 3 | 1 023 to 4 | 1 35 | 56 7 | 3 57 | 5 2 | 0 33 | 29 2 | 1 86 | 8 6 | 0 55 |
| 51 to 60 | 2 | 1 025 to 6 | 1 12 | 53 0 | 3 55 | 4 8 | 0 32 | 36 5 | 2 45 | 5 6 | 0 39 |
| 61 to 80 | 2 | 1 025 to 7 | 1 09 | 52 2 | 3 56 | 3 0 | 0 21 | 36 2 | 2 51 | 8 6 | 0 59 |
| 81 to 100 | 2 | 1 026 to 7 | 0 90 | 47 2 | 3 32 | 3 5 | 0 24 | 38 4 | 2 75 | 10 9 | 0 77 |
| 101 to 120 | 1 | 1 026 | 1 30 | 55 9 | 3 96 | 3 1 | 0 21 | 36 0 | 2 54 | 4 7 | 0 34 |
| 121 to 150 | 1 | 1 026 to 7 | 1 62 | 63 7 | 4 71 | 3 1 | 0 22 | 21 8 | 1 61 | 11 5 | 0 86 |
| 151 to 200 | 1 | 1 026 to 7 | 1 54 | 61 1 | 4 52 | 4 2 | 0 31 | 26 3 | 1 98 | 7 9 | 0 59 |
| 201 to 250 | 2 | 1 025 to 7 | 1 75 | 64 0 | 4 53 | 3 7 | 0 31 | 27 5 | 1 99 | 4 8 | 0 35 |

TABLE 4

EFFECTS OF THE INTRAPERITONEAL INJECTION OF THE VARIOUS RAT SERUM GLOBULINS ON THE SURVIVAL OF PARABIOSED ALBINO RATS

| Fraction injected prior to parabiotic union | Total No of pairs() | Male | Female | Success pairs () | Male | Female | Died during union () | Male | Female | Spontaneous separation () | Male | Female |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Albumin | (6) | 4 | 2 | (0) | 0/4 | 0/2 | (6) | 4/4 | 2/2 | (0) | 0/4 | 0/2 |
| Albumin-a1 | (11) | 7 | 4 | (2) | 1/7 | 1/4 | (5) | 4/7 | 1/4 | (4) | 2/7 | 2/4 |
| Alpha (littermates) | (11) | 7 | 4 | (4) | 2/7 | 2/4 | (5) | 4/7 | 1/4 | (2) | 1/7 | 1/4 |
| (nonlittermates) | (10) | 6 | 4 | (3) | 1/6 | 2/4 | (2) | 1/6 | 1/4 | (5) | 4/6 | 1/4 |
| Beta | (21) | 11 | 10 | (0) | 0/11 | 0/10 | (11) | 5/11 | 6/10 | (10) | 6/11 | 4/10 |
| Gamma | (14) | 4 | 10 | (2) | 1/4 | 1/10 | (8) | 3/4 | 5/10 | (4) | 0/4 | 4/10 |
| Fraction injected following union | | | | | | | | | | | | |
| Albumin | (10) | 5 | 5 | (3) | 1/5 | 2/5 | (5) | 3/5 | 2/5 | (2) | 1/5 | 1/5 |
| Albumin-a1 | (10) | 6 | 4 | (3) | 1/6 | 2/4 | (7) | 5/6 | 2/4 | (0) | 0/6 | 0/4 |
| Alpha | (11) | 4 | 7 | (2) | 1/4 | 1/6 | (5) | 2/4 | 3/6 | (4) | 1/4 | 3/6 |
| Beta | (10) | 6 | 4 | (2) | 0/6 | 2/4 | (5) | 4/6 | 1/4 | (3) | 2/6 | 1/4 |
| Gamma | (10) | 3 | 7 | (2) | 0/3 | 2/7 | (3) | 1/3 | 2/7 | (5) | 2/3 | 3/7 |

and also to separate spontaneously than did the males  Referring to successful untreated parabionts in TABLE 3, it is seen that the females survived in 37 5 per cent of the unions and the males in 15 0 per cent  From this, one can assume that any procedure that augments successful union or resists killing the parabionts during the period of union may be beneficial  Such a condition is found in the animals pretreated with the alpha-globulin fraction  Of the 11 littermate pairs so treated, 4 pairs went into successful parabiosis, and 2 pairs separated spontaneously, this is a survival of 54 5 per cent  With nonlittermates an even higher survival rate of 72 2 per cent was obtained  However, the actual percentage of successful parabiosis was higher in the littermate group (36 3 per cent as against 30 0 per cent)

Comparison of the findings with pretreatment with alpha-globulin and post-treatment instituted after parabiotic union demonstrated that the former method is most effective in survival of the animals and in obtaining successful parabiotic unions  However, the 2 successful pairs and the 4 separated pairs indicate a survival equal to that of the pretreatment procedure

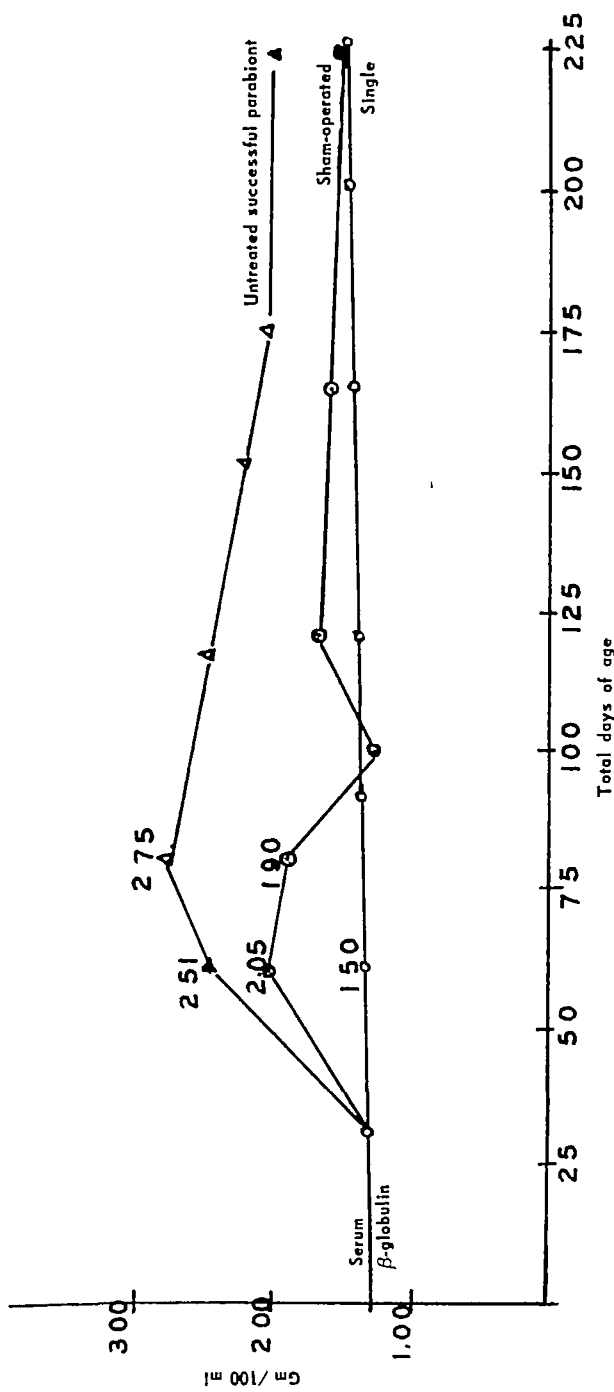
Of the other serum components given after parabiotic union was performed, gamma-globulin was found to be most effective in the survival of the animals

Although only 2 pairs were found in successful parabiosis, 5 pairs separated spontaneously to make a total of 70 0 per cent survival Albumin and albumin (plus alpha$_1$-globulin) resulted in the greatest number of successful unions (30 0 per cent in each group), yet the former had a 50 0 per cent survival rate, while the latter method of treatment resulted in only a 30 0 per cent survival rate

*Effects of serum component treatment on electrophoretic patterns* Comparison of the dominant significant changes found in the electrophoretic patterns in single, sham-operated, and successful untreated parabiotic pairs is confined to the albumin and beta-globulin levels The longer persistence of the serum beta-globulin effect is shown in FIGURE 1, which graphically demonstrates the early rise in the serum beta-globulin of sham-operated animals and the greater rise obtained in successful parabiotic pairs The numerals on the curves indicate the maximum absolute concentration of the particular component at the various ages analyzed

The electrophoretic concentration of the serum albumin and beta-globulin (in absolute amounts) fractions obtained by pretreatment and posttreatment of parabiotic animals with the alpha-globulins is shown in FIGURE 2 The base line obtained from single animals and successful untreated parabiotic pairs, also shown, serves to emphasize the deviations that occur with the treatment It should be noted that the absolute amounts of serum albumin of the experimental animals are higher than those found in the successful untreated parabionts, but lower than those obtained in single animals of the same age The serum beta-globulin titer of the experimental animals, on the other hand, is greater than the single animal level and less than that of the successful untreated parabionts

FIGURE 3 demonstrates the results obtained from the experimental use of the other fractions on the serum albumin and beta-globulin levels The titers obtained at the various ages for single animals serve as a base line The experimental group posttreated with gamma-globulin exhibits the only definite increase in the serum albumin above the control level It may be significant that its serum beta-globulin titer is almost equal to the single control level Based on the survival of the experimental parabiotic pairs, it did not give the best results, as only a 40 0 per cent survival rate was obtained Injection of albumin (plus alpha$_1$-globulin) immediately following parabiotic union gave this experimental group a 50 0 per cent survival rate The serum albumin level of this last experimental group was somewhat below that found for the single animals, and its serum beta-globulin level is found to be the next highest of all experimental groups It must be noted that these divergent effects in the albumin and beta-globulin levels resulted in only 20 0 per cent successful pairs for the postgamma-globulin treated animals and 30 0 per cent success in the parabiotic pairs posttreated with albumin (plus alpha$_1$-globulin) The most successful treatment was achieved by the pretreatment of prospective parabionts with alpha-globulins and resulted in almost 40 0 per cent successful pairs and over 50 0 per cent survival rate Reference to FIGURE 2 will show that the serum albumin of these experimental pairs was below the control level and the serum beta-globulin well above the single level



Untreated successful parabiont
Sham-operated
Single
Serum
β-globulin
Gm /100 ml
3.00
2.00
1.00
2.75
2.51
2.05
1.90
1.50
25
50
75
100
125
150
175
200
225
Total days of age


FIGURE 1

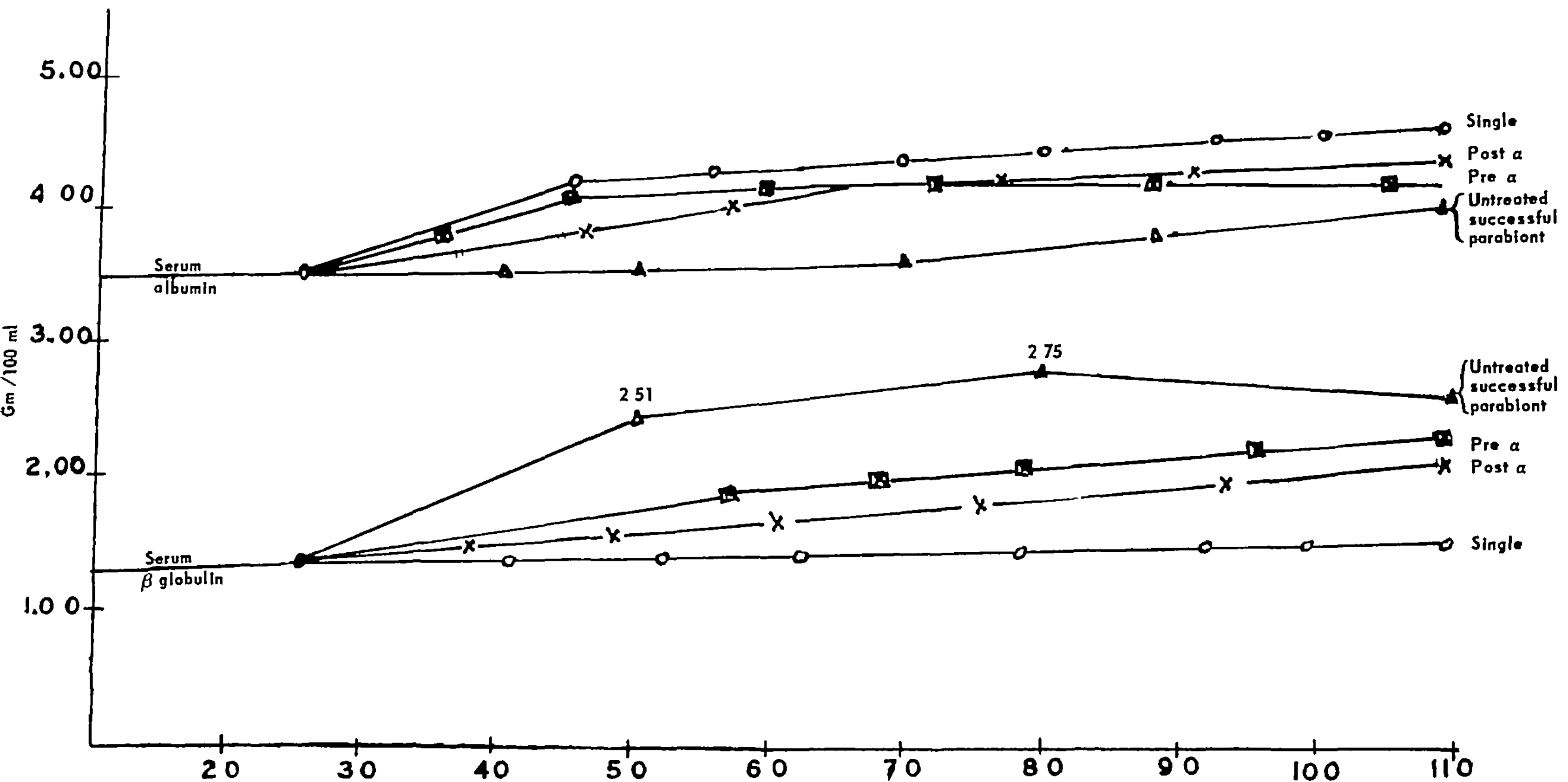

5.00
4.00
3.00
2.00
1.00
Gm /100 ml
Serum albumin
Serum β globulin
Single
Post α
Pre α
Untreated successful parabiont
2.75
2.51
Untreated successful parabiont
Pre α
Post α
Single
20
30
40
50
60
70
80
90
100
110
Total days of age

FIGURE 2

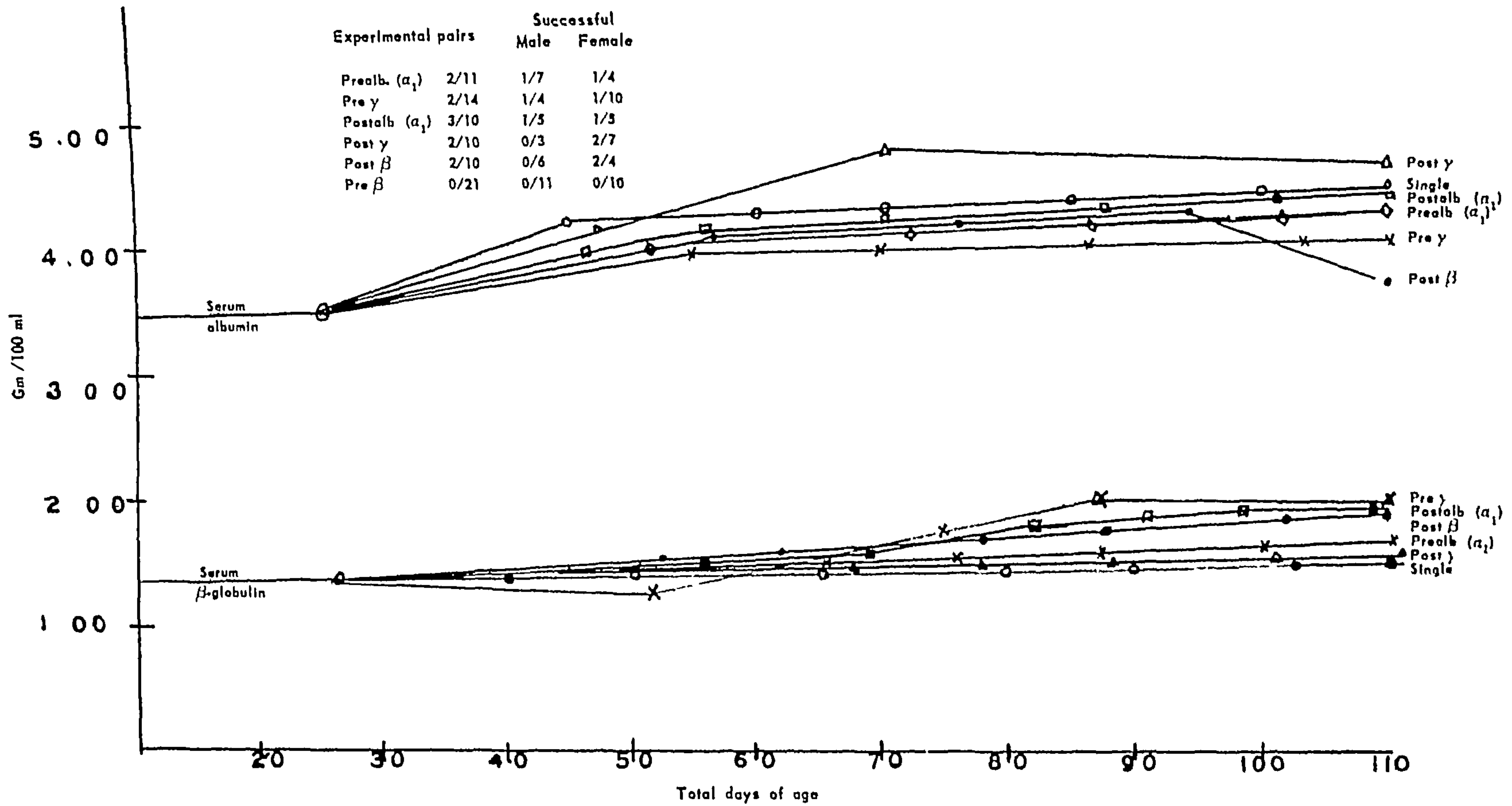

Experimental pairs
Successful
Male Female
Prealb. (a1) 2/11 1/7 1/4
Pre γ 2/14 1/4 1/10
Postalb (a1) 3/10 1/5 1/5
Post γ 2/10 0/3 2/7
Post β 2/10 0/6 2/4
Pre β 0/21 0/11 0/10
Post γ
Single
Postalb (a1)
Prealb (a1)
Pre γ
Post β
Pre γ
Postalb (a1)
Post β
Prealb (a1)
Post γ
Single
Serum albumin
Serum β-globulin
5.00
4.00
3.00
2.00
1.00
Gm/100 ml
20
30
40
50
60
70
80
90
100
110
Total days of age

FIGURE 3

*Discussion*

It is apparent from the data given that treatment with the globulins has a definite and discernible effect upon the survival of animals placed in parabiosis Purified rat albumin, if injected peritoneally 5 times before surgical union of littermates, will terminate in the death of all of the parabionts in about 2 weeks The same component in the same dosage, given following parabiotic union, will result in 30 0 per cent success and a total of 50 0 per cent survival Beta-globulin fraction injected prior to parabiotic union will give no successful pairs, and less than 50 0 per cent will survive The same component injected following parabiosis will facilitate the survival of 50 0 per cent of the pairs, of this total, 20 0 per cent will be in successful parabiotic union These findings suggest that the same component has different effects upon the organism, dependent upon the time of its administration

Albumin (plus alpha$_1$-globulin) fraction has an entirely different effect (based on time of administration) upon survival of surgically united animals If given as a preconditioner, there is a total survival of 54 5 per cent and, of this percentage 18 0 per cent will be in successful union When this same component is injected following parabiotic union, the total survival rate is only 30 0 per cent, and all of these pairs are in successful parabiosis This group differs from the lethal effects of preconditioning with purified albumin in that the majority of the animals did not die until the third to fourth week of union This finding again emphasizes the necessity of timing the injection of the material and also the beneficial effects derived from the presence of the alpha-globulins

Following the observation that the time of the injection as well as the material injected were of importance, a series of animals was subjected to alpha-globulin injections In the first group of 11 pairs of pretreated littermates (of which one pair was a male/female pair), 4 pairs went into successful union, and 2 pairs separated spontaneously, giving a survival rate of 54 5 per cent The male/female pair was found among the 36 3 per cent in successful parabiosis Pretreating 2 different litters born 2 days apart with the same materials and methods, and uniting these nonlittermates resulted in 80 0 per cent survival and 30 0 per cent successful pairs Injection of the alpha-globulins following parabiosis of littermates showed a 54 5 per cent survival rate, but only 18 0 per cent were in successful parabiosis The relatively high survival rate and the number of successful parabiotic pairs suggest that the alpha-globulins so condition the recipients that the low survival rate of untreated pairs is definitely enhanced The contrast of 23 2 per cent survival and 9 3 per cent successful parabiotic pairs in untreated animals, and the maximum of 80 0 per cent survival and as high as 36 3 per cent successful pairs in the treated experimental group makes this conclusion tenable

The effects of treatment with gamma-globulin are interesting and should be pursued further Pretreatment with this globulin results in 42 8 per cent survival, whereas injection following parabiotic union results in a survival rate of 70 0 per cent The percentage of successful pairs derived from the respective treatments was 15 0 and 20 0 per cent

Another facet of the problem that will bear further investigation was the

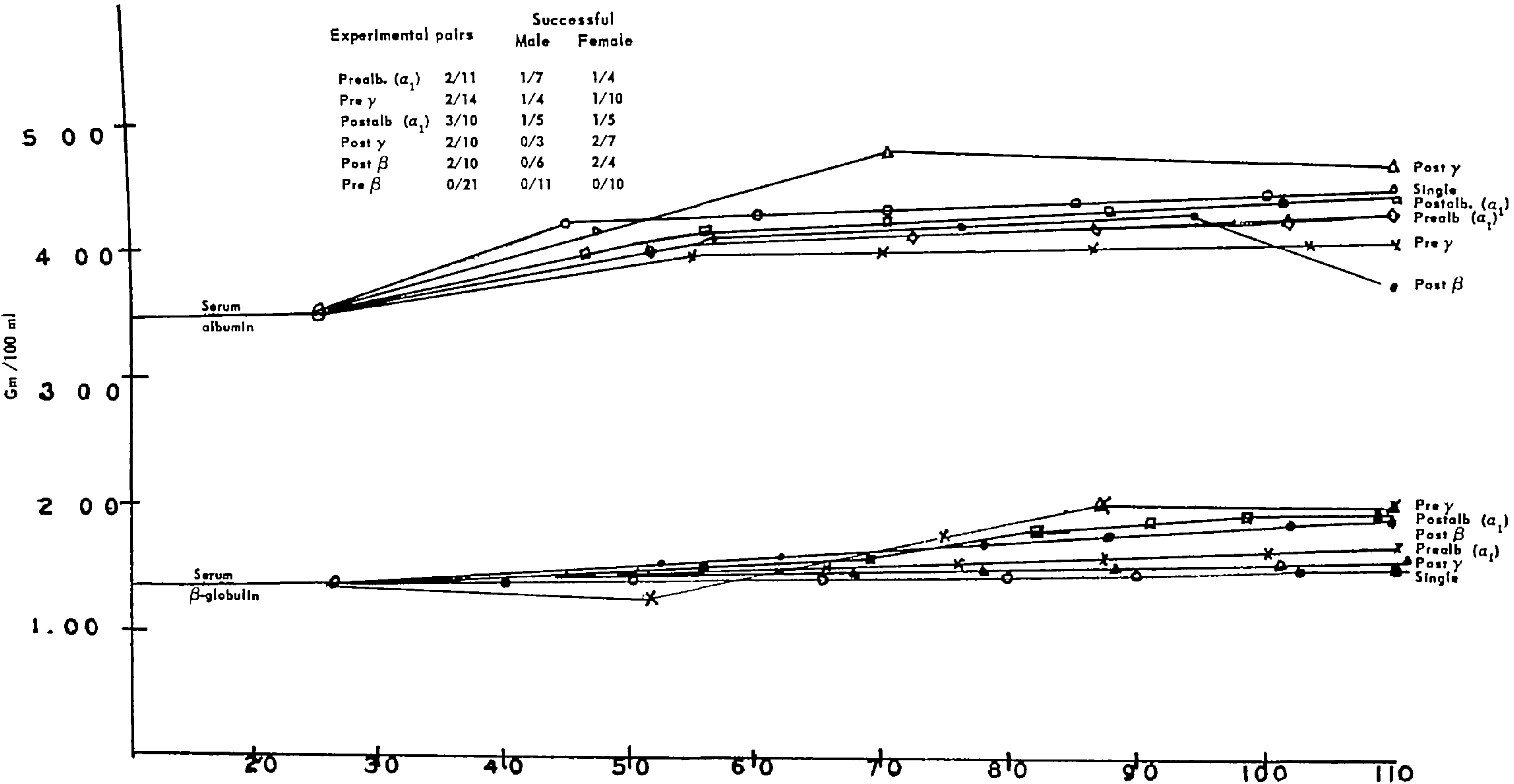

Experimental pairs
Successful
Male
Female
Prealb. ($a_1$) 2/11 1/7 1/4
Pre γ 2/14 1/4 1/10
Postalb ($a_1$) 3/10 1/5 1/5
Post γ 2/10 0/3 2/7
Post β 2/10 0/6 2/4
Pre β 0/21 0/11 0/10
Post γ
Single
Postalb. ($a_1$)
Prealb ($a_1$)
Pre γ
Post β
Pre γ
Postalb ($a_1$)
Post β
Prealb ($a_1$)
Post γ
Single
Serum albumin
Serum β-globulin
Gm /100 ml
5.00
4.00
3.00
2.00
1.00
20
30
40
50
60
70
80
90
100
110
Total days of age

FIGURE 3

tinuing parabiotic union results in significant increases in the serum beta-globulin levels of these animals

*Acknowledgment*

I express my appreciation for the able technical assistance of Michael A Kamrin during the summer of 1957 in carrying out this study

*References*

1 BERT, P 1862 Ann sci nat Sér IV **18** 86
2 SAUERBRUCH, F & M HEYDE 1908 Munch med Wochschr **55**: 153
3 HUFF, R L, R TRAUTMAN & D C VAN DYKE 1950 Am J Physiol **161**. 58
4 KAMRIN, B B 1954 J Dental Research **33**: 824
5 FINERTY, J C 1952 Physiol Revs **32**. 277
6 STEPHENSON, K L 1953 Plastic and Reconstr Surg **12**. 335
7 LOEB, L 1918 J Med Research **38** 393
8 SNELL, G D 1953 J Natl. Cancer Inst **14**. 691
9 EICHWALD, E J, C R SILMSER & N WHEELER 1957 Ann N Y Acad Sci **64**(5): 737
10 BILLINGHAM, R E, L BRENT & P B MEDAWAR 1955 Ann N Y Acad Sci **59**(3): 409
11 BUNSTER, E & R K MEYER 1933 Anat Record **57**. 339
12 PHILLIPS, R A, D D VAN SLYKE, P B HAMILTON, V P DOLE, K EMERSON, JR & R M ARCHIBALD 1950 J Biol Chem **183** 305
13 RAPPAPORT, F & G GEIGER 1934 Klin Wochschr **13**: 563
14 ANTWEILER, H J 1952 Die quantitative Elektrophorese in der Medizin Springer Berlin, Germany
15 GOLDSTEIN, J & J W ANDERSON 1957 J Biol Chem **224** 775
16 PILLEMER, L & M C HUTCHINSON 1945 J Biol Chem **158** 299
17 DARCY, D A 1957 Brit J Cancer **11** 137
18 COHN, M 1957 Ann N Y Acad Sci **64**(5) 859

finding that the female animals appear to have greater resistance to parabiotic death and more often go into successful union than do the male animals Of the 124 experimental pairs reported in this paper and the 43 untreated pairs, 90 pairs were male and 77 pairs were female The male/female pair is included in the male group In the untreated group, only 3 7 per cent of the males and 18 7 per cent of the females went into successful parabiotic union In the experimentally treated group as a whole, 6 4 per cent of the males and 12 1 per cent of the females resulted in successful unions

The processes by which the injected alpha-globulins exert their ameliorating effect on the animals prior to parabiotic union are unknown to this investigator A recent paper by Darcy[17] produces evidence that a substance normally present in the blood serum of rats and associated with the alpha-globulin fraction is increased five- to tenfold during the repair of tissues in wound healing or during rapid growth of tumors in these animals The present investigation has demonstrated by free electrophoresis that the serum of sham-operated and parabiotic animals contains a significant increase in the beta-globulin fraction It may be that the same substance is implicated in both instances Under the free electrophoresis conditions of this investigation, it migrates with the beta-globulin, while in Darcy's experimental set-up of gel electrophoresis it travels with alpha-globulins The consistent finding of similar electrophoretic response in both wound healing and parabiosis seems to indicate that there is some validity to this proposal

Of material consequence to this problem is the suggestion by Cohn[18] that the foreign antigen (death resulting from albumin and globulin treatments would suggest an antigen effect) may be considered as "an extracellular regenerated filter that prevents antibody from reaching the circulation or an intracellular inhibitor " The data presented in this paper suggest further that the alpha-globulins abrogate the homograft rejection reaction by blocking the capacity of the nucleoproteins to form antibody-producing active antigens Alternatively, the alpha-globulins may mobilize the entire antibody-forming capacity of the organism and prevent the homografted tissues from eliciting any further response

## *Summary*

(1) Parabiotic union is utilized as a method of testing homograft tissue tolerance Death of one or both animals of the union or spontaneous separation can be attributed to intolerance of the organisms to the homografted tissues

(2) Untreated albino rats placed into such parabiotic union show a survival rate of 25 0 per cent Of the total unions, 15 7 per cent separate spontaneously and 9 3 per cent go into successful and tolerant union

(3) Pretreatment of these animals with the alpha-globulin fraction of rat serum results in a survival rate of over 60 0 per cent, with 33 0 per cent of the total group in successful parabiosis

(4) Other fractions of the blood serum have varying effects upon survival and successful union of these animals, dependent upon the material itself and upon the time that it is introduced into the animals

(5) Wound healing in sham-operated animals and in the presence of con-

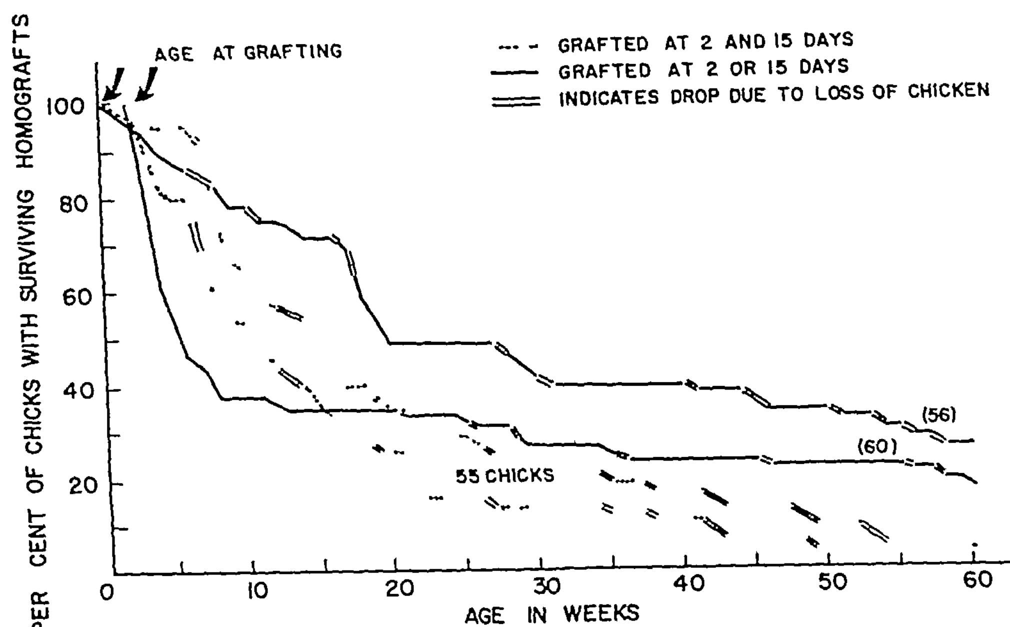



FIGURE 1 The altered behavior of 2-day and 15-day primary and secondary homograft on the same host, compared to controls chicks previously injected with blood from skin donor

vival of the primary graft in spite of slough of the secondary graft In this situation the supposedly tolerant host is obviously not tolerant to the same degree to 2 grafts from the same donor The reaction occurs first, not in the graft that has been in place the longer time, but in the graft that has been in place the shorter time The suggestion is very strong that there are different components present in the 2 grafts despite their origin from the same indiviudal Host tolerance alone does not adequately explain the alterations in the reactions that occurred

Another group of experiments involving tolerance induced by the method of embryonic blood transfusion indicated that, contrary to the hypothesis held by most investigators, induced tolerance is not at all entirely individual specific Thus, contrary to experiments reported by Billingham *et al*[4] we have found that, if a group of chick embryos of one breed are given a blood transfusion with blood from a chick embryo of another breed and at hatching are subjected to a skin homograft from a different donor than the blood donor, but of the same breed as the blood donor, the homografts will survive in an incidence approximately threefold that found in controls (FIGURE 2) Admittedly, the incidence is from one half to one third the incidence that occurs when the blood and skin donor are the same A similar situation obtains when the transfusion consists of blood pooled from 4 to 8 embryos of the same breed as the skin donor The initial incidence of graft survival in this situation is 5 to 10 times that in untreated controls (that is, through the first 10 weeks postgrafting) The (final) incidence of survival finally drops to only twice that found in the controls A further experiment has damaged the theory that tolerance under these experimental conditions is individual specific When the embryonic transfusion consisted of blood from one breed of chicken to another (interstrain trans-

# UNEXPECTED MANIFESTATIONS OF INDUCED TOLERANCE TO SKIN HOMOGRAFTS IN THE CHICKEN*

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Our study of the homograft problem has involved, for the most part, a scrutiny of the behavior of skin homografts in the chicken under a large variety of experimental conditions Some of our experiments have yielded either data that we find difficult to interpret in terms of the hypotheses offered by others or have given rise to confusion in our minds because of difficulty in formulating satisfactory hypotheses to allow interpretation of the findings

It is the purpose of this paper to present some of the problems that have arisen in the hope that others may be helpful in supplying satisfactory explanations In presenting these data, a minimum of experimental and technical details will be discussed and an attempt will be made to present the essentials of each problem in summary form The material and methods used have been reported elsewhere,[1, 2] and details of other experiments will be published [3]

Our basic principle in studying this problem in the chicken has been to involve relatively large numbers of subjects and to follow the grafts that survive for long periods of time By this means we believe our results have statistical significance and a relatively high degree of accuracy and reliability

The first point of confusion that we have encountered lies in the behavior of homografts obtained by simple interchange of skin squares between one-day-old chicks of different breeds The incidence of eventual permanent take is low but, in those chickens bearing a healthy homograft, successful repeat homografts from the same donor are rarely if ever obtained This situation appears to exist to a greater or less degree even when the experimental subjects have been subjected to induced tolerance by embryonic cross-transfusion of blood We are unable to explain the take of such homografts, while second homografts are sloughed, on the basis of a central failure of the host's immunological mechanism alone We believe strongly that graft adaptation must be an additional, prominent factor

A second experiment that appears to us to require more than the present theory of tolerance for a satisfactory explanation is as follows Chicks that have been subjected to embryonic cross-transfusion are homografted at 2 days posthatching The homografting is repeated between the same pairs at 15 days posthatching The 2 homografts are then observed until reaction occurs and the results compared with singly grafted but cross-transfused controls The long term result is that neither the 2-day grafts nor the 15-day grafts survive in as great an incidence as do singly grafted controls (FIGURE 1) In addition, the survival incidences of 2-day compared to 15-day homografts are definitely not the same on a given chick Invariably, when a reaction occurs the 15-day graft is sloughed first Thus there is a significant incidence of sur-

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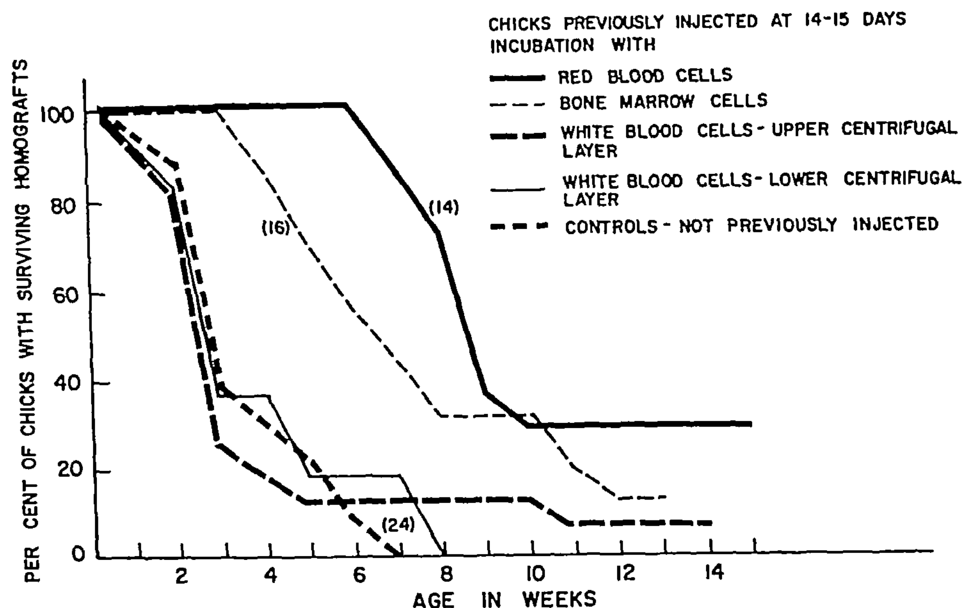



FIGURE 3 Behavior of various adult blood cell fractions in inducing tolerance in the chick survival of adult skin homografts on chicks previously injected with adult cells from homograft donor

sisted of multiple centrifugation and resuspensions of adult chicken blood so that four main fractions were obtained the serum, the RBC, the upper layer of the buffy coat, composed mostly of WBC of the lymphocytic and monocytic series, and the lower layer of the buffy coat, composed mostly of polymorphonuclear leukocytes In addition, an adult chicken bone marrow suspension was prepared The results of using these fractions in inducing tolerance were surprising to us (FIGURE 3) As would be expected, adult chicken serum had no effect We did find, however, in complete contradiction to Billingham's experiment[4] that the RBC suspension was far and above the most effective cell suspension of those tested in inducing tolerance Initially, 100 per cent of the adult skin homografts sustained a healthy take for 6 weeks There was then a relatively abrupt fall to a permanent incidence of about 35 per cent Next in order of effectiveness was the adult bone marrow suspension that was effective in 100 per cent of the grafts for 3 weeks By the end of 12 weeks, however, the permanent graft survival had dropped to about 15 per cent The cells in the upper layer of the buffy coat (lymphocytes) permitted a 15 per cent survival of the homografts for 10 weeks, but the permanent incidence of take then dropped to about 8 per cent The cells in the lower layer of the buffy coat produced an incidence essentially the same as the adult skin controls in that no grafts survived beyond 8 weeks It should be remembered that we have found that adult skin and 1-day-old skin behave very differently when placed on 1-day-old hosts[7] Whereas the 1-day-old skin invariably gives a permanent incidence of healthy homograft survival of about 10 per cent, adult skin grafts never survive significantly beyond the sixth week

Initial experience with adult whole blood transfusions into chick embryo showed a much higher mortality to the embryos than when embryonic blood was transfused It quickly became apparent that the cells in the buffy coat

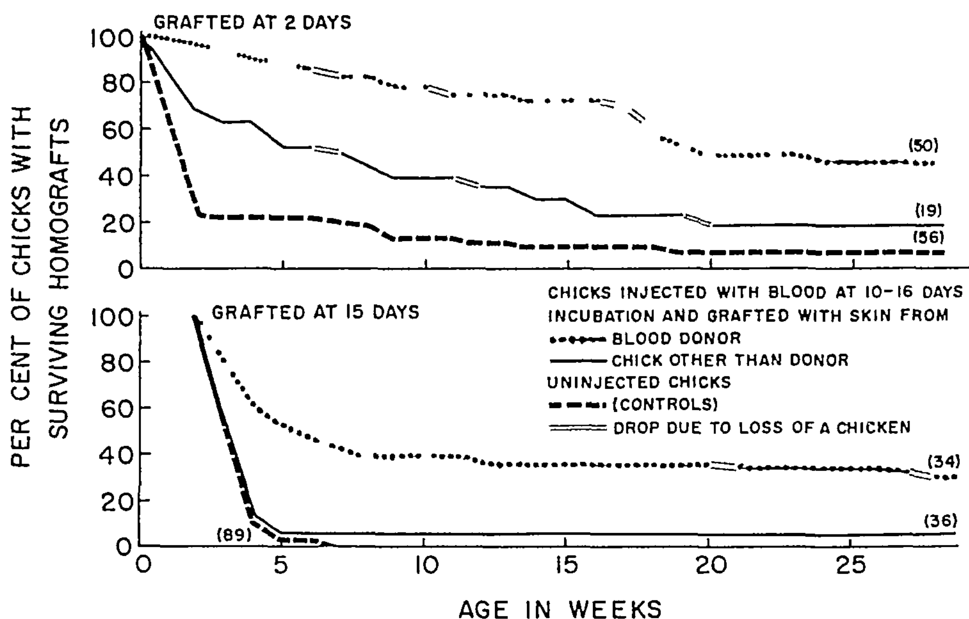



FIGURE 2 Graphic summary of experiments indicating the nonspecificity of tolerance in the chick survival of skin homografts from blood-donor and nondonor birds

fusions) and when, in addition, skin was grafted from chicks of the same breed as the blood recipient (intrastrain grafting) it was quite apparent from the results that the interstrain transfusion induced in the recipient a significant degree of tolerance to the intrastrain homografts

With regard to the above experiments, it is pertinent to mention that the nonspecific tolerance obtained does not seem to be in any way related to inbreeding or shared antigens, since control animals from all groups involved invariably show no evidence of relationship when subjected to skin homografting at two weeks of age One other constant finding, which we have noted in all of the thousands of chick grafts we have performed, is the absence of signs of compatibility between the chicks of a pair that are cross-homografted Permanent take in both homografts of a pair is almost never encountered The 5 to 10 per cent permanent incidence of take in the Danforth preparations[5] and in the Billingham preparations[6] is almost invariably comprised of an incidence of homograft survival in one member of the pair rather than in both

In the course of the work it became apparent that the use of embryonic blood donors for inducing tolerance imposed limitations with regard to the amount of blood available, possibilities as to fractionation, and the required survival of the donor We became increasingly interested in determining the comparable behavior in tolerance induction of the various blood cell fractions Consequently it was necessary to turn to the adult chicken as the blood and skin donor One of us (P I T ) conceived and conducted a group of experiments under these conditions, the results of which we find both confusing and provocative Details of these experiments will be reported elsewhere

The work of Billingham *et al* [4] has indicated that red blood cells (RBC) are not effective in inducing tolerance in the chicken and that the white blood cell (WBC) fraction is the most effective in this influence Our experiments con-

Most fascinating to us was the finding that the lower layer, or polymorphonuclear fraction, of the buffy coat of centrifuged adult chicken blood was not only ineffective in our hands in producing tolerance (as judged by permanent homograft survival) but was also highly lethal to the embryo This phenomenon may constitute another manifestation of homologous disease, the appearance of which has been so discouraging to hopes for clinical applications in this problem The fact that this lethal effect can be abolished by washing the cells at room temperature may be a clue to some aspect of the cause of the homologous disease phenomenon If cells are available that can induce tolerance but are free of the taint of homologous disease, perhaps our clinical hopes may be allowed slight rejuvenation

The motivation in the direction of the research in our laboratory is basically clinical in nature At the present time, given a choice between finding a practical clinical application of homologous transplantation and further pursuit of an understanding of the basic nature of the reaction, we should undoubtedly choose the former As clinicians we must proceed on the premise that the homograft problem actually can be solved for clinical application, or our efforts in the field are hardly justified Consequently we feel that the basic question that must be answered concerns the matter of whether tissue specificity in terms of homotransplantation is alterable

Although there is an overwhelming amount of evidence that tissue specificity is under genetic control in the absence of experimental intervention, we cannot help but conclude that there is evidence (including some of our own work) in favor of the alterability of tissue specificity We also believe that a major argument for this premise is seen in the most common experiment that nature constantly performs, namely, the homografting between sperm and ovum, which seems to occur with a relatively high incidence of success Nevertheless, it seems that there are two schools of thought with regard to the nature of tissue specificity One school intimates, without complete proof in our opinion, that tissue specificity factors are under complete control of genes and that, consequently, alterability in the individual may well be impossible, since tissue specificity factors are built into each cell of the individual from the time of the first cell division of the fertilized ovum The other school is composed of pupils who, for the most part, are confused as to mechanisms and explanations but who, as a group, believe that tissue specificity may be a factor that is added to the tissue as the individual develops The alterability in the embryo and infant with regard to tissue transplantation is an established fact, although its mechanism is far from explained Alterability in the adult involves, at present, major destructive forces and is attended by other major problems such as homologous disease as a price for limited success

*Summary and Conclusions*

(1) Certain groups of experiments, the results of which were either unexpected or in disagreement with similar work by others, are briefly presented

(2) Evidence is presented to indicate that at least the tissue specificity characteristics in the skin of a newly hatched chick are less distinctive and more

of adult blood were responsible for this mortality. Embryos injected at the tenth to twelfth day of incubation were seen to continue normal development until the eighteenth or nineteenth day of incubation and then to succumb Autopsy of these embryos showed only one abnormality a marked hypertrophy of the spleen that showed normal splenic structure on histological examination This toxic factor was found to exist only in the adult WBC found in the lower layer of the buffy coat, the mortality induced averaged about 80 per cent in the dosage used The autopsied embryos invariably showed only the one abnormality, namely, marked splenic hypertrophy This lethal effect found in these lower-layer WBC could be abolished by washing in saline at room temperature, it was preserved in spite of washing the lower-layer WBC in saline at 10° C The buffy coat of embryonic centrifuged blood, whethes of the upper or lower layer, is nontoxic to embryos in the same dosages as those used with adult cells Very recently Simonsen[8] has published a study of such splenomegaly produced by injection of adult spleen cells and leukocytes

*Discussion*

The results of the first two groups of experiments mentioned above indicate to us very strongly that host tolerance to a given homograft is only one major factor in determining graft survival It would appear obvious that the tissue specificity constituents of the homograft are at least of equal importance These experiments seem to necessitate the conclusion that tissue specificity factors in a given individual undergo definite alterations during the maturation of that individual Apparently, under the conditions of these experiments, graft adaptation is a factor in the continued survival of these homografts

The third group of experiments concerning the nonspecificity of induced tolerance may call for a basic alteration of the theory of tolerance as originally put forth by Billingham *et al* [4] We tend to interpret these results as indicating a progressive maturation of both the antibody-producing mechanism and the constituents that are capable of provoking an immune response in another animal During the process of development and differentiation of the individual it would seem that tissue specificity factors may well differentiate according to the same progression that occurs in other components of the organism, being more labile and less narrowly determinate in earlier than in later stages Thus complete individuality in terms of completely crystallized tissue specificity may not be present in the chick until about the fourteenth day after hatching

We consider the final group of experiments on induced tolerance in which adult blood cell fractions were used to be most provocative In the first place our results again appear to be in direct contradiction to those of Billingham *et al* [4] in that we found adult RBC to be by far the most effective in inducing tolerance We are not surprised that RBC are effective under these circumstances since the RBC of the chicken are nucleated It will be noted further that a relatively permanent degree of tolerance was apparently produced by these adult cells which, according to present knowledge, have a life span of not more than 20 to 30 days and definitely do not have the capacity of reproducing by cell division Consequently, the notion that cell chimerism is a necessary prerequisite for induced tolerance cannot apply in this experiment

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## *Summary and Conclusions*

(1) Certain groups of experiments, the results of which were either unexpected or in disagreement with similar work by others, are briefly presented

(2) Evidence is presented to indicate that at least the tissue specificity characteristics in the skin of a newly hatched chick are less distinctive and more

subject to alteration than the skin of the same individual once immunological maturation has occurred

(3) Evidence is presented showing that induced tolerance is not exclusively individual specific

(4) Evidence is presented that indicates that (a) adult chicken RBC are highly effective in inducing tolerance, (b) that adult chicken WBC are not, and (c) that adult chicken polymorphonuclear leukocytes contain a factor, removable by washing in saline at room temperature, that is highly lethal on the eighteenth or nineteenth day of incubation when injected into chick embryos on the tenth to twelfth day of incubation The only abnormality shown by the dead embryo is splenomegaly

(5) The implications of these data in relation to the nature of the homograft reaction are discussed

*References*


1 TERASAKI, P I & J A CANNON 1957 Proc Soc Exptl Biol Med **94** 103–107
2 CANNON, J A, P I TERASAKI & W P LONGMIRE, JR 1957 Am J Surg **94**· 303–312
3 TERASAKI, P I, J A CANNON & W P LONGMIRE, JR To be published
4 BILLINGHAM, R E, L BRENT & P B MEDAWAR 1956 Phil Trans Roy Soc Ser B **239** 357–414
5 DANFORTH, C H & F FOSTER 1928–1929 J Exptl Zool **52** 443
6 CANNON, J A, P TERASAKI & W P LONGMIRE, JR 1957 Ann Surg **146** 278
7 CANNON, J A, R A WEBER & W P LONGMIRE, JR 1954 Ann Surg **139** 468–472
8 SIMONSEN, M 1957 Acta Pathol Microbiol. Scand **40** 480–500